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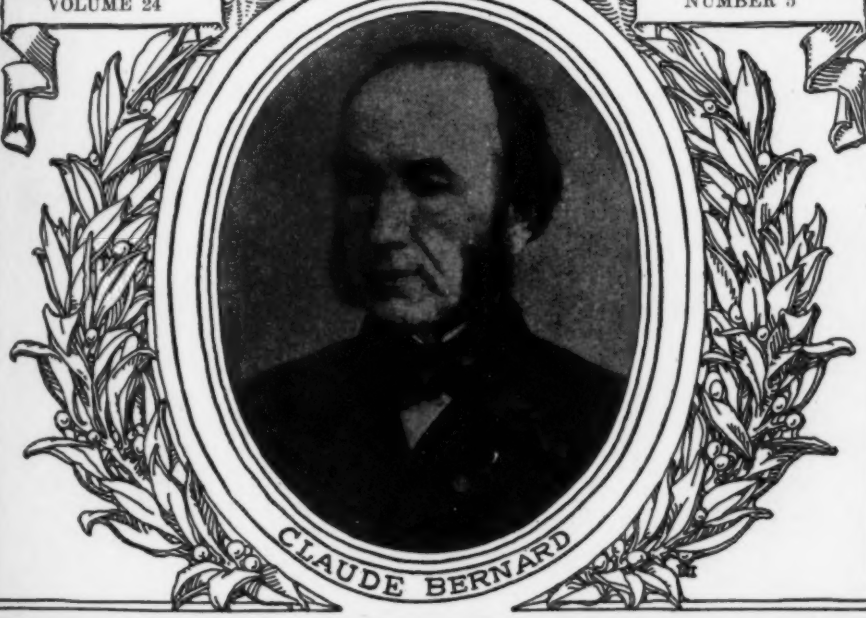

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THE INFLUENCE OF THIAMINE, RIBOFLAVIN, PYRIDOXINE, AND PANTOTHENIC ACID DEFICIENCIES ON NITROGEN METABOLISM [1]

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(Received for publication June 10, 1942)

While there is appreciable information concerning the influence of avitaminoses on fat and carbohydrate metabolism, the literature offers as yet relatively very little knowledge concerning nitrogen metabolism in vitamin deficiency diseases. This is particularly true with respect to the experimental rat.

In 1925 Morgan and Osburn reported on the effect of vitamin A deficiency on the nitrogen partition of the urine of the rat. In 1932 Sure and Kik found only a slight rise in non-protein nitrogen of the blood of the albino rat in a deficiency of the vitamin B complex. Recently Griffith and Mulford ('41) found that a deficiency of dietary choline and of the labile methyl supply in young rats produces a marked elevation of the non-protein nitrogen of the blood, which was coincident with renal hemorrhagic degeneration. The report of Lewinson ('38) on changes in non-protein nitrogen of the blood of dogs in B-avitaminosis cannot be critically reviewed, since his diets consisted of natural foods and were deficient in several dietary factors. Recently Schaefer, McKibbin and Elvehjem ('42) reported that in severe states of pantothenic acid deficiency in the dog there is a rise in the non-protein nitrogen of the blood.

[1] Research paper no. 745 Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

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The inadequate knowledge concerning the nitrogen metabolism in deficiencies of various components of the vitamin B complex and the recent development in this laboratory of procedures for blood (Sure and Wilder, '41) and urine analysis (Sure, Ford, Theis and Goldfischer, '41) stimulated this investigation, the results of which are presented in tables 1 to 5, inclusive. The partition of the non-protein nitrogen of the blood and urine was studied in thiamine, riboflavin, pyridoxine, and pantothenic acid deficiencies in the albino rat.

All of these studies were carried out with the paired feeding method of experimentation, according to the technique previously described (Sure and Dichek, '41). Since the litter mate control animals were restricted to the same amount of food consumed by the avitaminotic animals, the plane of nutrition was eliminated as a factor that possibly could have complicated the exogenous metabolism.

DIETARY TECHNIQUE USED FOR PRODUCING DEFICIENCIES OF VARIOUS COMPONENTS OF THE VITAMIN B COMPLEX

The composition of all rations is given in table 1.

Thiamine deficient diets

The thiamine investigation was conducted on two types of diets. Ration 9 contained autoclaved yeast and beef as sources of the various components of the vitamin B complex other than thiamine. The control animals received in addition 20 μg. thiamine daily. This ration, supplemented occasionally with 0.05 to 0.1 μg. thiamine daily per animal, allowed prolonged maintenance, thus making it possible to produce a chronic state of deficiency. For this reason, urinary nitrogen excretions as well as blood changes in non-protein nitrogen constituents were studied on this as well as on the more purified ration 10. Each animal received daily as a supplement to ration 10 the following vitamin doses: 20 μg. riboflavin, 20 μg. pyridoxine, 0.5 mg. nicotinic acid, 6 mg. choline chloride, 100 μg. calcium pantothenate, and 150 mg. of rice

polish factor II (Supplee, Bender and Kahlenberg, '40). The control animals also received 20 μg. thiamine daily. All animals receiving no cod liver oil in their rations were given 2 drops halibut liver oil weekly as a source of vitamins A and D. A sufficiency of vitamin E for growth was furnished by the butterfat or hydrogenated cottonseed oil in the rations.

TABLE 1

Percentage composition of rations.

COMPONENT	RATION						
	9	10	13	14	15	16	17
Casein [1]	16		18				
Casein [2]		18		18	18	18	18
Agar-agar		2		2			
Agar-agar [3]					2		
Nicotinic acid				0.05			
Cod liver oil			2		2	2	2
Butterfat		10		10			
Hydrogenated cottonseed oil [4]	9		8		3	8	8
Salts no. 351 [5]	4		4				
Salts no. 1 [6]		4					
P. and H. salts [7]				4	4	4	4
Autoclaved yeast [8]	7						
Autoclaved beef [9]	8						
Sucrose [3]					71	68	
Dextrin	56	66		65.95			
Dextrin [10]			68				
Cerelose							68

[1] Supplied by Atterbury Bros., New York City, and purified by washing for 10 days with acidulated water and then by several washings with 25% ethanol at room temperature.

[2] Borden's purified casein, supplied under the trade name "Labco."

[3] Hot alcohol extracted.

[4] Crisco.

[5] Hubbell, Mendel and Wakeman ('37).

[6] Sure ('41).

[7] Phillips and Hart ('35).

[8] Fleischmann's, autoclaved for 5 hours at 15 pounds' pressure.

[9] Autoclaved for 6 hours at 20 pounds' pressure.

[10] Dextrin carried an 80% alcoholic extract of 25 gm. rice polishings in 100 gm. of ration.

Riboflavin deficient diets

The riboflavin investigation was carried out on rations 13 and 14. The dextrin in ration 13, which is a modification of the diet of Day and Darby ('38), carried an 80% alcoholic extract of 25 gm. rice polishings per 100 gm. of ration as a source of the various components of the vitamin B complex with the exception of riboflavin. This diet was supplemented with 10 μg. thiamine and 10 μg. pyridoxine per animal per day and the control animals received in addition 20 μg. riboflavin daily. Ration 14 was supplemented with the same vitamin doses as ration 10 (previously outlined), the control animals being allowed 20 μg. riboflavin daily.

Pyridoxine deficient diets

Pyridoxine deficiency was produced on ration 15, which is a modified diet of Antopol and Unna ('39). The daily vitamin supplements were the same as used by the latter investigators. Our experience with the production of pyridoxine deficiency in the rat corresponded with the recent observations of Lepkovsky and Krause ('42), that is, we encountered a mild avitaminosis, manifested by slow growth without the accompanying dermatitis. Substitution of the purified sucrose by commercial dextrose [2] did not change the character of the avitaminosis. Lepkovsky and Krause ('42) now find that, if weaned rats are first depleted for 10 to 12 days on a diet deficient in the vitamin B complex, a greater incidence of dermatitis can be produced later by pyridoxine deficiency. Since dermatitis is also produced by pantothenic acid deficiency (Richardson, Hogan and Itschner, '41) and riboflavin deficiency (Sure and Dichek, '41), it is questionable, as pointed out by Lepkovsky and Krause, if dermatitis or acrodynia is a reliable expression of uncomplicated pyridoxine deficiency. That we were actually dealing with an avitaminotic state was evident from the accelerated growth the control animals made when allowed food ad libitum.

[2] Cerelose.

Pantothenic acid deficient diets

Pantothenic acid deficiency was produced on rations 16 and 17. As a supplement to this ration, the following daily vitamin doses were administered: 40 μg. thiamine, 40 μg. riboflavin, 40 μg. pyridoxine, 6 mg. choline chloride and 0.5 mg. nicotinic acid. The control animals received in addition 200 μg. calcium pantothenate daily. On ration 16, which is a modification of the diet by Unna ('40), only slow growth was the index of pantothenic acid deficiency, the characteristic symptoms described by Unna being absent. The change of ration 16 to ration 17, which consisted in the replacement of sucrose by dextrose,[3] together with different doses and kinds of vitamin supplements, resulted in the production of severe expressions of pantothenic acid deficiency. The daily vitamin supplements to ration 17 were as follows: 20 μg. thiamine, 20 μg. riboflavin, 20 μg. pyridoxine, 3 mg. p-aminobenzoic acid, 10 mg. inositol, 6 mg. choline chloride, and 0.5 mg. nicotinic acid. The control rats also received 100 μg. calcium pantothenate daily. Two to 3 weeks following the change from ration 16 to 17, the sores around the mouth, rusty spots on the fur and the "blood-caked" whiskers observed by Unna ('40) were regularly encountered. According to McElroy, Salomon, Figge and Cowgill ('41), the so-called bloody exudates of the nose which settle on the whiskers causing them to cake are composed not of blood but of coproporphyrin. After being on ration 17 for 6 to 7 weeks, animals collapsed suddenly when the plane of nutrition was quite adequate, an experience we have had recently with riboflavin deficiency (Sure and Dichek, '41). In this respect our experiences are in accord with those recently reported by Supplee, Bender and Kahlenberg ('42) on rats, and by Schaefer, McKibbin and Elvehjem ('42) on dogs. The sudden collapse may be due to adrenal hemorrhage and necrosis, and kidney and cardiac damage (Supplee and associates, '42; Mills et al., '40).

[3] See footnote 2, page 408.

TECHNIQUE USED IN BLOOD STUDIES

With the aid of the Evelyn macro-micro photoelectric colorimeter (Evelyn, '37) a technique was developed for micro determinations of non-protein nitrogen, urea nitrogen, creatine and creatinine. One milliliter of caudal blood was found sufficient for all the determinations of the non-protein constituents of the blood. In order to study the effect of the severity of the vitamin deficiency state on the concentration of such blood constituents in the living animal, it was necessary to pool the blood from several pairs of animals subjected to the same treatment and showing similar symptomatology. Enough blood could be secured by caudal bleeding from three animals weighing 60 to 70 gm. Such a technique has made it possible for the first time to follow the nitrogen partition of the blood of the living small animal. The details of such procedures are given elsewhere (Sure and Wilder, '41), but since the blood uric acid determination was not described, a brief outline is given for analysis of this constituent.[4]

This investigation involved a large number of blood and urine determinations. Space, therefore, does not permit us to show detailed results. All the blood data were grouped and averaged, and the urinary data were subjected to a statistical analysis according to Student's method, as outlined in the text by Paterson ('39).

NITROGEN METABOLISM IN THIAMINE DEFICIENCY

Influence of thiamine deficiency on changes in non-protein constituents of the blood

Blood samples were collected once weekly from six pairs of thiamine deficient animals and their controls on ration 9,

[4] Blood uric acid determination. To 1 ml. of the blood filtrate add 9 ml. of distilled water. Add 5 ml. of 5% sodium cyanide. Mix well and add 1 ml. of Benedict's uric acid reagent. Mix and place centrifuge tubes in incubator at 40°C. for 45 minutes. Place in ice bath for 15 minutes and then centrifuge for 5 minutes. Decant and read in Evelyn macro photoelectric colorimeter, using a 660 filter. From a previously calibrated curve obtained from various concentrations of pure uric acid crystals the values of blood uric acid are obtained.

from the sixth to the seventy-seventh day of vitamin depletion periods. No changes in the concentration of the non-protein constituents of the blood were apparent until the fortieth day. From then on there were 40 to 100% increases in the total N.P.N. and 30 to 50% increases in the urea nitrogen in the thiamine deficient animals. The changes in the uric acid were variable and in the creatine [5] and preformed creatinine [5] the differences were insignificant. In another group consisting of four pairs on ration 9 the blood changes were studied from the forty-second to the sixty-ninth day of vitamin depletion. From the forty-second day to the completion of the experiment there were 40 to 50% increases in the total N.P.N. and urea nitrogen, and about 24% increase in the preformed creatinine in the thiamine deficient rats. Similar observations were made on another group of four pairs of animals on ration 9 and on eleven pairs on ration 10. There were always marked increases in total N.P.N. and urea nitrogen and moderate increases in preformed creatinine in the avitaminotic rats, the earliest rise having been observed on the thirty-second day of vitamin depletion.

In order to obtain a more complete picture of the nitrogen metabolism, eleven pairs of animals were sacrificed in various states of thiamine deficiency, the summarized results of which are presented in table 2. It will be noted that even in the mild chronic state, and in the mild state of deficiency associated with small losses of body weight there are large increases in total N.P.N. and in urea nitrogen; also appreciable increases in preformed creatinine, the changes in uric acid and creatine being negligible. In the terminal states of avitaminosis, as indicated by polyneuritis and convulsions, only two pairs out of six showed increases in the total N.P.N. and urea nitrogen. The changes in the preformed creatinine were variable in this group. There were large increases in two avitaminotic animals, small increases in two other animals, and a decrease

[5] The creatine is expressed as creatinine and is obtained by subtracting the creatinine values before hydrolysis from the total creatinine obtained after hydrolysis.

TABLE 2

Influence of various deficiencies of the vitamin B complex on distribution of non-protein nitrogen of the blood of the albino rat.

AVITAMINOSIS	RATION	ANIMALS	NUMBER OF PAIRS	AVERAGE VITAMIN DEPLETION PERIOD	TOTAL NON-PROTEIN NITROGEN	UREA	URIC ACID	PRE-FORMED CREATININE	CREATINE	AVITAMINOTIC STATE
				days	*mg./%*	*mg./%*	*mg./%*	*mg./%*	*mg./%*	
Thiamine	9	P [1]	3	45	34.7	17.8	2.10	1.83	7.53	Early vitamin
		RC [2]			32.0	9.0	2.72	1.80	6.75	depletion state
Thiamine	9	P	5	100	62.6	20.2	2.16	1.53	10.12	Mild chronic
		RC			48.2	16.8	2.11	0.98	9.82	state
Thiamine	9	P	3	77	70.6	45.3	3.90	3.40	2.83	Mild, slow loss
		RC			46.3	23.6	3.47	2.20	3.13	of body weight
Thiamine	9 and 10	P	6	98	69.3	30.0	2.54	2.28	6.11	Polyneuritis and
		RC			63.8	28.8	2.89	1.97	6.81	convulsions
Riboflavin	13 and 14	P	16	113	57.7	19.3	1.72	1.38	5.19	Advanced state
		RC			48.4	15.3	1.93	1.62	5.00	of deficiency
Pyridoxine	15	P	5	30th to	57.1	23.0	2.65	2.47	6.19	Mild state of
		RC		106th	51.2	18.8	1.93	2.58	3.92	deficiency
Pyridoxine	15	P	4	48th to	58.8	23.1	2.08	2.55	5.36	Mild state of
		RC		121st	48.1	20.2	1.69	2.43	3.94	deficiency
Pantothenic	16 and 17	P	6	61	43.0	20.0	2.35	2.80	3.25	Advanced state
acid		RC			47.5	21.5	1.90	2.65	3.95	of deficiency
Pantothenic	16 and 17	P	4	68	49.0	19.5	2.10	2.50	3.25	Terminal state
acid		RC			38.0	14.5	2.75	2.15	4.00	of deficiency

[1] P = pathological animal.

[2] RC = restricted control animal.

in one. During this period the plane of nutrition is reduced to almost complete starvation, which is also true for the control animals restricted to the same amount of food; therefore, it is possible that starvation may have complicated the blood picture.

Influence of thiamine deficiency on partition of nitrogen of urine

The partition of nitrogen in the urine was carried out on ten pairs of animals on diets 9 and 10 during metabolism periods ranging from 7 to 27 days, according to the technique recently described elsewhere (Sure, Ford, Theis and Goldfischer, '41). The urine was collected and analyzed daily, with the exception of Sundays. Monday's samples, therefore, represented 48 hours' excretion. Space does not permit reproduction of the numerous daily and bidaily records. Only average figures are consequently presented for all the animals covering the average vitamin depletion periods. The urinary data on riboflavin, pyridoxine, and pantothenic acid deficiencies were similarly treated. Since, with the exception of creatine, the advance of avitaminosis did not always show proportionate differences in the nitrogen constituents of urine, the average figures are representatives of marked changes, if any occurred regularly.

Because of differences in amounts of total nitrogen excreted, it was necessary, in order to make comparisons between thiamine deficient and control animals, to calculate the excretions of all nitrogenous constituents in the urine as percentage of total nitrogen. Therefore, the uric acid, preformed creatinine, and creatine figures were recalculated and expressed as uric acid nitrogen, preformed creatinine nitrogen, and creatine nitrogen.

It is evident from table 3 that thiamine deficiency is followed by large excretions of ammoniacal nitrogen and a marked creatinuria, as shown by both creatine and preformed creatinine elimination in urine. On diet 10 the creatine output

TABLE 3

Influence of various deficiencies of the vitamin B complex on partition of nitrogen in the urine of the albino rat. Summarized average daily excretions per rat for all animals used in metabolism studies.

(All the nitrogen constituents of the urine are expressed in milligrams.)

CATEGORY OF INTEREST		AVITAMINOSIS			
		Thiamine	Riboflavin	Pyridoxine	Pantothenic acid
Ration		9 and 10	13 and 14	15	16 and 17
Nos. of pairs		10	8	11	12
Average metabolism period (days)		15	29	10	19
Total nitrogen	P[1]	114.9	89.6	131.8	103.9
	RC[2]	122.5	83.4	117.3	91.9
Urea nitrogen	P	84.6	65.0	107.5	76.3
	RC	95.2	61.9	92.4	66.4
Per cent of T. N.[3]	P	72.4	72.3	81.2	72.5
	RC	76.5	73.1	78.7	70.4
Ammonia nitrogen	P	6.9	5.8	4.5	8.7
	RC	3.2	3.7	4.7	8.1
Per cent of T. N.	P	6.6	6.7	3.5	8.4
	RC	3.1	4.6	4.1	9.4
Allantoin nitrogen	P	13.2	11.7	10.4	12.9
	RC	16.1	12.0	10.9	12.6
Per cent of T. N.	P	12.1	13.0	8.4	13.1
	RC	13.8	14.6	9.5	14.6
Uric acid nitrogen	P	0.63	0.59	0.51	0.84
	RC	0.63	0.58	0.56	0.79
Per cent of T. N.	P	0.55	0.67	0.39	0.84
	RC	0.61	0.69	0.48	0.94
Preformed creatinine nitrogen	P	2.44	0.54	0.97	0.83
	RC	1.54	0.69	0.96	0.82
Per cent of T. N.	P	2.11	0.60	0.77	0.83
	RC	1.23	0.82	0.81	0.95
Creatine nitrogen	P	1.30	0.56	0.91	0.65
	RC	1.04	0.76	0.72	0.63
Per cent of T. N.	P	1.16	0.63	0.69	0.62
	RC	0.81	0.91	0.61	0.69

[1] P = pathological or avitaminotic animal.
[2] RC = restricted control animal.
[3] T. N. = total nitrogen.

during the terminal stages of the thiamine deficiency was frequently found to be 100% greater than that in the litter mate control animals.

Since in the later stages of thiamine deficiency there is considerable catabolism of body tissue (Sure and Dichek, '41), during which periods there are considerable losses of creatine from muscle tissues, it was necessary to recalculate the excretions of creatine and preformed creatinine on the basis of

TABLE 4

Summarized data on the influence of a deficiency of various components of the vitamin B complex on partition of nitrogen of the urine of the albino rat.[1]

Nitrogen partition of urine expressed as per cent of total nitrogen.

CATEGORY OF INTEREST	AVITAMINOSIS			
	Thiamine	Riboflavin	Pyridoxine	Pantothenic acid
Nos. of pairs	10	8	11	12
Urea nitrogen				
Per cent decrease	5.3	1.1		
Per cent increase			3.2	3.0
Ammonia nitrogen				
Per cent decrease			14.6	10.6
Per cent increase	113.2	45.7		
$\frac{D}{E_D}$ [2]	12.43 [3]	3.80 [3]	2.95 [4]	1.17
Allantoin nitrogen				
Per cent decrease	12.3	10.9	14.6	10.3
$\frac{D}{E_D}$ [2]	0.54	3.80 [3]	1.64	3.88 [3]
Uric acid nitrogen				
Per cent decrease	9.6	2.9	18.8	10.6
$\frac{D}{E_D}$ [2]	0.37	0.68	4.09 [3]	0.15

[1] Results are expressed as changes in the avitaminotic animals compared with litter mate controls.

[2] $\frac{D}{E_D}$ represents t values which equal the mean difference divided by standard error of mean differences, according to Student's method of statistical analysis (Paterson, '39).

[3] Indicates that the experimental results are statistically very significant.

[4] Indicates that the experimental results are statistically significant.

100 gm. of body weight. Such figures eliminated the disturbing factor of changes in body weight influencing the creatine-creatinine metabolism. The average daily urinary excretions for the ten pairs of animals during the vitamin depletion periods were as follows: Preformed creatinine: avitaminotic, 2.42 mg.; control, 1.41 mg., or an increase for the thiamine deficient animals of 71.6%. Creatine: avitaminotic, 1.38 mg.; control, 1.03 mg., or an increase for the avitaminotic animals

TABLE 5

Summarized data on the influence of a deficiency of various components of the vitamin B complex on creatine-creatinine excretion in urine, calculated on basis of body weights.

Average daily excretions per 100 gm. body weight.

AVITAMINOSIS		NOS. OF PAIRS	CREATINE NITROGEN			PREFORMED CREATININE NITROGEN		
			Mg.	Per cent increase	$\frac{D}{E_D}$	Mg.	Per cent increase	$\frac{D}{E_D}$
Thiamine	P [1]	10	1.38	34.9	1.47	2.42	71.7	8.60 [4]
	RC [2]		1.03			1.41		
Pyridoxine	P	8	0.66	17.9	3.23 [3]	0.75	25.0	2.46 [3]
	RC		0.56			0.60		
Riboflavin	P	11	0.67	21.8	2.39 [3]	0.81	5.2	1.09
	RC		0.55			0.77		
Pantothenic acid	P	12	0.55	25.0	3.51 [3]	0.73	15.8	3.65 [3]
	RC		0.44			0.63		

[1] Pathological animal.

[2] Restricted control animal.

[3] Indicates that the experimental results are statistically significant.

[4] Indicates that the experimental results are statistically very significant.

of 34.9%. When the results of creatine and creatinine excretions, as related to body weights, were subjected to Student's method of statistical analysis, only the large excretions of creatinine were found to be significant. The $\frac{D}{E_D}$ or t value for one error in a thousand, when the number of variants is 10, is 3.169 (Paterson, '39). Actually, the t value found was 8.601 (table 5), which is beyond any possibility of error.

The figure 1.465 for the creatine excretions indicates that, although the average figures show an increase of 34.9%, there were too many fluctuating results in obtaining such average, for the chances for error are one in about seven; hence, the results when correlated with fluctuations in body weight, are statistically insignificant. The t value of 12.432 for 113.2% increase in excretion of ammonia (table 4) shows beyond any doubt that the results are free from any possibilities of error. On the other hand, the small decreases in allantoin and uric acid excretions have no statistical importance.

The marked excretions of preformed creatinine and retention of this constituent in the blood may be correlated with muscular atrophy during catabolism, as indicated by large losses of body weight in thiamine deficiency. The cause for the increases in both total N.P.N. and urea nitrogen is not as clear. The increases in blood total N.P.N. in choline deficiency (Griffith and Mulford, '41) and in pantothenic acid deficiency (Schaefer, McKibbin and Elvehjem, '42) are explained on the basis of kidney damage from hemorrhages. The question arises whether in thiamine deficiency there may be sufficient kidney damage to interfere with its normal excretory function. According to Hoobler ('28), one of the outstanding symptoms of infantile beriberi, as observed in the Philippines, is oliguria. There is a reduction in the amount of urine to the extent that the "diapers are wet once or twice a day and then with only scanty amounts of urine." Such symptomatology certainly should result in retention of non-protein constituents of the blood. In the rat, however, no noteworthy microscopic changes were found in the kidney in uncomplicated vitamin B_1 deficiency (Sure, Thatcher and Walker, '31). Since the diets used by the latter investigators carried autoclaved yeast which contains appreciable amounts of undestroyed thiamine, there may have been enough of this vitamin in the ration to prevent kidney damage. Further work on the influence of thiamine deficiency on the pathology of the kidney of the rat is, therefore, necessary to establish its relation to reten-

tion of total N.P.N. and urea nitrogen in the blood. On the other hand, the increase of these constituents of the blood in thiamine deficiency may very well be due to anhydremia produced in this avitaminosis (Sure and associates, '29 a, '29 b; Stucky and Rose, '29). It is possible that actually there may be a decrease in blood uric acid in thiamine deficiency, but being masked by the anhydremia, no noteworthy changes are apparent. Since in the majority of cases the increase in blood preformed creatinine was accompanied by a decrease in creatine, it is possible, as recently demonstrated by Bloch and Schoenheimer ('39) that the latter constituent was transformed into the former.

The marked increases in excretion of ammoniacal nitrogen may be explained by the attempt of the avitaminotic animals to neutralize acids developed in the acidosis associated with vitamin B_1 deficiency (Sure and Smith, '29).

NITROGEN METABOLISM IN RIBOFLAVIN DEFICIENCY

Influence of riboflavin deficiency on distribution of non-protein nitrogen constituents of the blood

Eight pairs of rats fed ration 14 were sacrificed at various periods of riboflavin depletion ranging from 90 to 154 days, and five pairs on ration 13 were sacrificed after 74 to 107 days of vitamin depletion. The pathological changes observed were dermatitis, alopecia, keratitis, and premature senility. That these cases represented advanced stages of riboflavin deficiency is apparent from the observations that many control animals had gained two to three times as much body weight as the litter mate riboflavin deficient rats on the same amount of food intake. Yet, there were no noteworthy changes in the non-protein nitrogen constituents of the blood (table 2) when the results were subjected to a statistical analysis. The indications of slight rises in the total N.P.N. and in urea nitrogen have no significance, since the results for sixteen pairs of animals were inconsistent. An analysis of pooled blood from three pairs in terminal stages of riboflavin deficiency also showed no significant changes. One of the avitaminotic animals of this

group died the day following the termination of this experiment; another died 14 days following the end of this experiment. It may be concluded, therefore, that even in advanced and terminal stages of riboflavin deficiency there are no noteworthy changes in the various non-protein constituents of the blood.

Influence of riboflavin deficiency on partition of nitrogen of urine

The partition of nitrogen in the urine was carried out on eight pairs of rats on ration 14, summarized average results of which are presented in table 3. The metabolism periods ranged between 8 and 72 days. In seven cases out of eight the period of study was not less than 16 days, and since the depletion periods were sufficiently advanced, the changes found are representative of what takes place in this avitaminosis. That there are large increases in excretion of ammonia is quite evident, and the results are statistically very significant, the possibility of error being less than one in a thousand. The figures on excretion of creatine and preformed creatinine are, however, misleading unless correlated with changes in body weight. For instance, Daniels, Hutton and Neil ('38), in the use of the creatinine-height coefficient as an index of nutrition of children, make use of the body weight. The creatinine coefficient in man is defined by Beard and Jacob ('39) "as the amount in milligrams of creatinine or creatinine nitrogen excreted daily per kilogram of body weight." Since in the rat riboflavin plays a significant role in economy of food utilization (Sure and Dichek, '41), and frequently the control animals will weigh 100 to 150% more than the avitaminotic litter mates, lower excretion of both preformed creatinine and creatine may be anticipated in the riboflavin deficient rats. When calculated, however, on the basis of average daily excretions per 100 gm. body weight, the results were as follows: Preformed creatinine: avitaminotic, 0.66 mg.; control, 0.56 mg., or an increase of

17.9% for the riboflavin deficient animals. Creatine: avitaminotic, 0.75 mg.; controls, 0.60 mg., or an increase of 25% for the riboflavin deficient animals. Statistically, the results are significant for both the creatine and creatinine values. In the case of the former, the chances for error are about 5%. In the case of the latter, the chances for error are less than 1%. It may be concluded, therefore, that there is a moderate creatinuria in riboflavin deficiency in the albino rat. The large excretions in ammonia suggest an acidosis in this avitaminosis, as has been found in thiamine deficiency (Sure and Smith, '29). There is also a small but statistically significant reduction in allantoin excretion in this avitaminosis.

NITROGEN METABOLISM IN PYRIDOXINE DEFICIENCY

Influence of pyridoxine deficiency on distribution of non-protein nitrogen of the blood

The blood changes in this avitaminosis were followed in five pairs of animals from the thirtieth to the one hundred and sixth day of vitamin depletion, according to the technique described earlier in the paper. As already stated, the only signs of pyridoxine deficiency observed were inadequate gains in body weight.

Although no regular changes were apparent either in the total non-protein nitrogen or urea nitrogen of the blood, the marked changes in both uric acid and creatine were consistent throughout the whole 76-day vitamin depletion period. This is also true of the next group, consisting of four pairs, the blood changes of which were followed from the forty-eighth to the one hundred and twenty-first day of pyridoxine deficiency. Averaging the results from these nine pairs of animals for a depletion period of 75 days, the avitaminotic rats showed an increase of 31.4% in blood uric acid and an increase of 47.0% in blood creatine. Whether retention of such blood constituents takes place earlier in pyridoxine deficiency we have not determined.

Influence of pyridoxine deficiency on partition of nitrogen in the urine

The partition of nitrogen in the urine was carried out on two groups of animals. The first group consisted of five pairs, the urine of which was pooled. The second group consisted of six individual pairs. The results of these experiments are summarized in table 3. The pyridoxine deficient rats excreted daily an average of 0.91 mg. creatine while the control animals excreted 0.72 mg. or an increase of 26.4%. Since, however, the avitaminotic rats excreted daily more total nitrogen than the controls, calculated on the per cent of the total nitrogen, the increase of creatine excretion for the pyridoxine deficient animals is only 13.1%. When, however, the changes in body weight are taken into consideration and the average daily excretions are expressed on the basis of 100 gm. body weight, the results were as follows: Creatine: avitaminotic, 0.67 mg.; controls, 0.55 mg., or an increase for the pyridoxine-deficient animals of 21.8%. Preformed creatinine: avitaminotic, 0.81 mg.; controls, 0.77 mg., or an increase for the avitaminotic of 5.2%. Because of the retention of blood creatine, the origin of the urinary creatinine is most probably the blood creatine, the latter being derived from muscle metabolism. Such transformation is in line with the recent results of Bloch and Schoenheimer ('39). If pyridoxine is essential for normal muscle metabolism, it should prove of beneficial therapeutic use in certain types of myopathies. Two clinical reports show the therapeutic value of pyridoxine in such diseases (Antopol and Schotland, '40; Spies, Bean and Ashe, '39).

The reduction in allantoin excretion has no statistical value, since chances for error are about one in six.

The marked retention of blood creatine and uric acid and the reduction in urinary excretion of uric acid (table 4) suggest that there may be a disturbance in kidney function in pyridoxine deficiency.

NITROGEN METABOLISM IN PANTOTHENIC ACID DEFICIENCY

Influence of pantothenic acid deficiency on distribution of non-protein nitrogen of the blood

The concentration of the non-protein constituents of the blood was followed in one group of three pairs of animals from the ninth to the forty-ninth day of vitamin depletion, and in another group of three pairs from the fourteenth to the forty-second day of vitamin depletion. The changes in these blood constituents were insignificant. In table 2 are submitted average results on animals changed from ration 16 to ration 17, which was followed by advanced states of deficiency, ending in the death of several avitaminotic rats either the same day or several days after the last sampling of blood. One pair was sacrificed when the avitaminotic animal was in a dying condition. It will be noted that only in the terminal stages of pantothenic acid deficiency do we find a noteworthy rise in the total non-protein nitrogen and urea concentration of the blood. The changes in the rest of the blood constituents were negative. Schaefer, McKibbin and Elvehjem ('42) also found a marked rise in the total N.P.N. in severe states of pantothenic acid deficiency in the dog but none in mild states.

Influence of pantothenic acid deficiency on partition of nitrogen of urine

Nitrogen partition of the urine was studied in twelve pairs of animals on rations 16 and 17. In a number of cases the changes in the urinary excretion of nitrogenous constituents were followed until the animals died. In this avitaminosis, as in the case of riboflavin deficiency, we have had complications of great differences in body weights between pathological and control animals. Calculated on the basis of 100 gm. body weight, the average daily excretions of creatine and preformed creatinine were as follows: Creatine: avitaminotic, 0.55 mg.; controls, 0.44 mg., or an increase for the pantothenic acid deficient rats of 25%. Preformed creatinine: avitaminotic, 0.73

mg.; controls, 0.63 mg., or an increase for the avitaminotic rats of 15.8%. The t values indicate that the chances of error are less than one in one hundred for both the creatine and creatinine values. Hence, it may be concluded that in pantothenic acid deficiency there is a moderate creatinuria. There is also a small but statistically significant reduction in allantoin excretion in this avitaminosis.

The question arises whether specific gravity determinations of the blood would have been of assistance in the interpretation of the blood data. In the case of thiamine deficiency, this has already been done (Sure and associates, '28; Stucky and Rose, '29) and such correlations were made earlier in the paper. It is very doubtful, however, whether blood concentration or dilution complicated the picture of the non-protein constituents of the blood in riboflavin, pyridoxine, or pantothenic acid deficiencies for the following reasons: (a) In riboflavin deficiency the blood changes were negative, so information on concentration or dilution would have been of no importance; (b) in pyridoxine deficiency only two blood constituents increased, namely, uric acid and creatine, but not the main constituents, i.e., total N.P.N. and urea. If anhydremia were present, the latter constituents would have increased, and if edema were present, the same blood components would have decreased; (c) in pantothenic acid deficiency changes were found only in the terminal stages in two components. If either anhydremia or edema were present, the condition would have been evident much earlier during the progress of the avitaminosis.

SUMMARY AND CONCLUSIONS

In thiamine deficiency there are large excretions of ammonia, creatine, and preformed creatinine in the urine. The creatinuria may be rated as marked. While there is a significant relationship between large excretions of preformed creatinine and body weight, there is no such definite relationship between creatine elimination and body weight in this avitaminosis when the data are examined statistically. There

is a great increase of total non-protein nitrogen and urea in the blood even in mild chronic states of thiamine deficiency.

In riboflavin deficiency there are large excretions of ammonia in the urine and a moderate creatinuria. The latter was manifested by excretions of both creatine and preformed creatinine when changes in body weight were taken into consideration during the vitamin depletion periods. There is also a small reduction in allantoin excretion in the urine. These changes were noted in advanced states of deficiency.

In pyridoxine there is a mild creatinuria, a reduction in urinary excretion of uric acid, and a marked retention of creatine and uric acid in the blood. These observations were made in mild states of this avitaminosis.

In pantothenic acid deficiency there is a moderate creatinuria, and a small reduction in allantoin excretion in the urine. The increases in total non-protein nitrogen and urea of the blood were noted only in the terminal stages of this avitaminosis.

We wish to acknowledge our appreciation to Merck & Company for their contribution of crystalline thiamine, riboflavin, pyridoxine, calcium pantothenate, and choline chloride used in this study.

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INHIBITION OF THE SYMBIOTIC SYNTHESIS OF B COMPLEX FACTORS BY SULFONAMIDES

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ONE FIGURE

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Black, McKibbin and Elvehjem ('41) have found that the addition of sulfaguanidine to a B complex-free basal diet supplemented with the available crystalline components of the B complex, results in a depressed rate of growth of young rats. This effect was promptly ameliorated by feeding a liver extract, and could be prevented by feeding para-aminobenzoic acid. They suggest that sulfaguanidine inhibits the growth of certain microorganisms which may normally synthesize essential unidentified factors of the B complex. These factors were presumably supplied by the liver extract. Dann ('41) has also demonstrated that the incorporation of sulfaguanidine in the diet adversely affects the growth of rats fed no source of the B complex other than the crystalline vitamins.

Daft, Ashburn, Spicer and Sebrell ('42) have reported that rats fed a B complex-free diet containing 1% sulfaguanidine plus the crystalline factors of the B complex, develop hyaline sclerosis and calcification of the arteries. Whether the condition is due to the toxicity of the drug or to the deficiency of the unidentified B complex factors is not yet established.

The present study was designed to indicate whether the effect of sulfaguanidine on the growth of rats is due to a direct action on the metabolism of the animal or to an indirect

action through preventing a symbiotic synthesis of unidentified factors by the intestinal flora.

It seems probable that animals fed the B complex-free diet supplemented with the crystalline factors of the B complex are supplied with essential unidentified components of the B complex through synthesis by their intestinal flora. If this assumption is true, then the feces of these animals should contain these factors. Furthermore, if the effect of sulfaguanidine in the diet is to inhibit the growth of the organisms responsible for the synthesis of these factors in the intestinal tract, then the feces of such animals would be a poor source of the factors. On this premise we set up the following experiment.

EXPERIMENTAL

White rats of the Sprague Dawley strain, weighing from 60 to 75 gm., were divided into twelve groups. The first four groups were fed the B complex-free basal diet [1] either alone or modified by the incorporation of certain sulfonamide derivatives as indicated in the table. Each animal was individually fed a supplement of crystalline vitamin having the following composition: thiamine 20 μg., riboflavin 40 μg., pyridoxine 20 μg., calcium pantothenate 100 μg., nicotinic acid 0.5 mg., inositol 1 mg., choline 5 mg., and biotin [2] 0.2 μg. per day.

The remaining eight groups were placed on the vitamin B complex-free basal diet containing 0.5% sulfaguanidine. These groups were depleted of the B complex vitamins for 2 weeks. At the end of this period they were all given the same daily supplements of crystalline vitamins as groups I to IV, and further supplementary feedings of feces or natural B complex sources as indicated in the table.

The feces from nine animals in each of the groups I to IV were collected and the feces from each group for each week were combined, dried at low temperature, and ground. Each

[1] B complex-free basal diet having the following percentage composition: sucrose, 71; casein, 20; salts, 4; agar, 3; A, D, E oil, 2 (contains 888 U.S.P. units vitamin A, 177 U.S.P. units vitamin D, and 11 mg. alpha tocopherol per gram of oil).

[2] S M A — 1000 × concentrate.

week's collection was then divided into five equal parts, and one part was fed, by admixing with the basal ration, to each animal of the appropriate groups as indicated in the table. By this schedule a week intervened between the final collection of feces for the week and feeding the sample, during which time the feces were prepared for incorporating in the diet.

RESULTS

The following table presents both the details of the experimental plan and the growth response of the groups.

TABLE 1

The effect of sulfonamides on the symbiotic synthesis of a B complex factor(s).

GROUP NO.	BASAL DIET	SUPPLEMENTS	NO. OF ANIMALS	AVERAGE GAIN IN 4 wks.	AVERAGE GAIN IN 7 wks.
				No depletion period	
				gm.	gm.
I	B complex-free + 0.5% sulfaguanidine	Pure B vitamins	9	63	81
II	B complex-free + 0.5% sulfanilamide	Pure B vitamins	9	58	88
III	B complex-free + 0.5% sulfadiazine	Pure B vitamins	9	62	75
IV	B complex-free	Pure B vitamins	9	83	129
				After 2 weeks depletion	
V	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + feces of group I	5	63	92
VI	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + feces of group II	5	88	124
VII	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + feces of group III	5	76	102
VIII	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + feces of group IV	5	99	138
IX	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + 250 mg. yeast	8	106	152
X	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + 50 mg. yeast	8	88	123
XI	B complex-free + 0.5% sulfaguanidine	Pure B vitamins + 50 mg. yeast extract	8	102	143
XII	B complex-free + 0.5% sulfaguanidine	Pure B vitamins	9	58	83

The effect of incorporating 0.5% sulfaguanidine in the diet of animals receiving only the available pure components of the B complex is apparent from the growth curves for groups I and IV in figure 1. Feeding adequate amounts of yeast or yeast extract to animals receiving a basal diet with 0.5% sulfaguanidine permits a marked improvement in the rate of

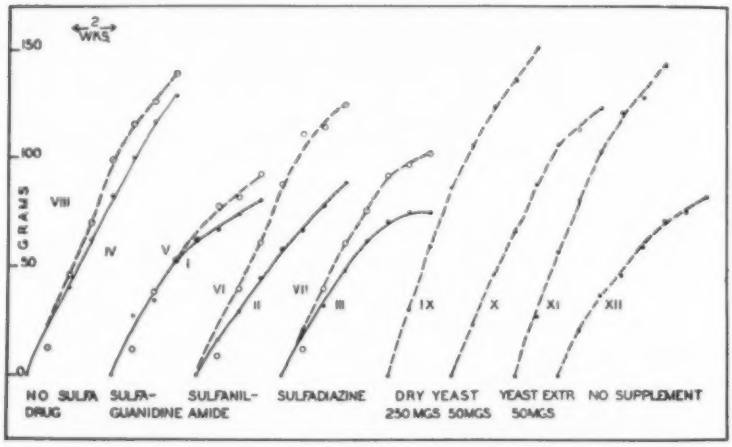

Fig. 1 The solid lines (.———.) indicate the growth of the undepleted groups of animals fed all the pure vitamins except para-aminobenzoic acid and the B complex-free diet containing the designated sulfonamides at a 0.5% level. Feces from each group graphed on a solid line were fed, respectively, to each corresponding group delineated on the adjoining broken line.

The broken lines (.- - - - -.) indicate the growth of the depleted groups receiving the same pure vitamin supplements and the same basal diet containing 0.5% sulfaguanidine. Further supplements of feces, as previously indicated, or of yeast or yeast extract, were given in daily doses as indicated in the chart.

growth (cf. groups IX, X, XI, and XII). This result might be attributed either to detoxication of the sulfaguanidine or to supplementation with unidentified factors of the B complex.

A critical evaluation of the results presented in the first two sets of curves (fig. 1) obtained with groups VIII, IV, V, and I leads one to believe that the effect of sulfaguanidine is to inhibit the growth of certain strains of the intestinal flora

which normally synthesize unidentified factors of the B complex.

Groups V and VIII received exactly the same diet (0.5% sulfaguanidine) and the same pure vitamin supplement as group XII, except that group V animals were fed in addition the feces from group I, and group VIII the feces from group IV. It is obvious from the growth curves that the feces from group IV animals contain factors which permit sulfaguanidine-fed animals to grow as well as those similarly fed but supplemented with yeast. On the other hand the feces from group I animals had little, if any, of these factors present.

The third and fourth pairs of curves in figure 1 present the results obtained by incorporating 0.5% sulfanilamide and sulfadiazine in the basal diets of groups II and III, respectively, and in feeding the feces of these two groups to the sulfaguanidine-fed animals in groups VI and VII, respectively. The responses indicate that both sulfanilamide and sulfadiazine have a toxic effect, but that neither is as effective as sulfaguanidine in inhibiting the growth of the particular organisms responsible for the synthesis of the unidentified B complex factors. The sulfanilamide apparently has less effect in inhibiting these particular organisms. Such a result might be anticipated from the data of Lawrence and Sprague ('41), whose study indicated that sulfanilamide was less effective in reducing fecal counts than was sulfaguanidine, sulfathiazole, or sulfapyridine.

The direct toxic effect of both drugs was further confirmed by our observations at autopsy. All of the animals fed 0.5% sulfadiazine or sulfanilamide exhibited marked hyperemia and enlargement of the thyroid which was more pronounced in the sulfadiazine groups, and the kidneys of a number of the animals revealed the yellow radiation on macroscopic examination. Histological examination revealed that these thyroids contained very little colloid material and the acini were hyperplastic. The thyroids of animals fed 0.5% sulfaguanidine were not enlarged and exhibited little evidence of hyperplasia.

Sections of the lung, heart, liver, kidney, stomach, pancreas, spleen, and cecum from several animals of each of groups I, IV, VIII, XI, and XII, as well as the sulfadiazine and sulfanilamide animals were also prepared and studied.

Examination of sections from animals fed sulfaguanidine did not reveal the hyaline sclerotic changes in the walls of the small arteries, which Daft et al. ('42) reported as occurring in young rats fed a B complex-free diet containing 1% sulfaguanidine and supplemented with pure vitamins for periods of 62 to 192 days. We did, however, observe small clear areas in the muscular coat of coronary arteries of most of the rats examined, and similar changes in the arteries of other organs. There was no evidence of inflammatory changes around the clear areas. These changes were observed in the tissues of the animals from group IV, which were not fed sulfaguanidine, as well as in the tissues of the animals receiving the diet with 0.5% sulfaguanidine. There was more amorphous basophilic material in the renal tubules of some of the animals receiving sulfanilamide and sulfaguanidine than in the controls. Findings on other tissues were essentially negative. All of the animals had received the experimental diet for 75 days at the time they were autopsied. Another lot of animals were sacrificed after 138 days on their respective diets and the histological picture remained the same.

DISCUSSION

There is apparently a lack of correlation between the development of hyaline sclerosis, hyperplastic thyroids and the deficiency of the unidentified factors produced by feeding sulfonamides. Daft et al. ('42), feeding a diet containing 1% sulfaguanidine, produced a deficiency of the unidentified factor, and observed hyaline sclerosis and calcification of small arteries. According to the results of Mackenzie, Mackenzie and McCollum ('41), this diet should also have induced a hyperplasia of the thyroid, although there was no comment on either the presence or absence of these symptoms in the report by Daft et al. ('42).

In the present experiment animals fed diets with 0.5% sulfaguanidine with or without a deficiency of the unidentified factor did not develop either hyaline sclerosis of the arteries or hyperplasia of the thyroid. The animals fed 0.5% sulfanilamide or sulfadiazine did develop hyperplastic thyroids, but no hyaline changes in the arteries.

It is possible that the hyaline changes in the arteries are a specific effect of the 1% level of sulfaguanidine, or that some minor difference in the experimental procedure may account for the failure of our animals to develop hyaline sclerosis and calcification of the arteries.

The evidence seems clear that there are certain, as yet unidentified, factors present in yeast which are essential for the normal growth of rats, and normally synthesized in their intestines. The synthesis of these factors can usually be repressed by feeding the animals 0.5% sulfaguanidine in the diet. The sulfaguanidine apparently inhibits the growth of only certain types of organisms. Preliminary fecal counts, made after the animals had been fed a B complex-free diet containing 0.5% sulfaguanidine supplemented with the pure vitamins for 8 weeks, indicated that no notable decrease in the total count had been maintained, although there appeared to be a difference in the composition of the flora.

We have observed, in previous studies, that a certain percentage of individuals will grow well in spite of receiving a B complex-free diet containing 0.5% sulfaguanidine supplemented only with the crystalline components of the vitamin B complex. This observation suggests that certain species of organisms capable of synthesizing the unidentified factors, which are not commonly found in the rat intestinal flora, and which are not sensitive to sulfaguanidine, had become established in the intestines of these animals found refractory to the sulfaguanidine diet. Another possible explanation is that the critical strains, through conditioning, have become immune to the drug (Vivino and Spink, '42).

Woolley ('42) has demonstrated that the spontaneous cures of inositol deficiency symptoms in mice are due to the syn-

thesis of inositol by certain types of organisms in the intestinal flora. He found that the flora of mice which exhibit inositol deficiency symptoms does not synthesize appreciable amounts of this compound.

It has been shown by McElroy and Goss ('40) and others that the flora of ruminants synthesizes sufficient quantities of the B complex components to supply significant amounts of these factors to the host. There is evidence that the rat, equipped by nature with a large cecum, is supplied with certain factors of the B complex through intestinal synthesis, and that the mouse may be supplied with inositol in a similar manner. However, there are undoubtedly other species not so fortunately endowed by nature, which will not be able to rely on the intestinal flora to synthesize the essential factors and must obtain these factors in the diet. It is probable that dogs and man will be in the latter category, since neither has a large cecum or comparable "fermentation vat."

Further work on the types of organisms susceptible to sulfaguanidine therapy and a study of their ability to synthesize the unidentified factors will be helpful in elucidating this problem.

SUMMARY

The inclusion of 0.5% sulfaguanidine in the diet of rats fed the available crystalline factors of the B complex results in a depressed growth. This effect can be counteracted by feeding such natural sources of the B complex as yeast and yeast extract. It is also prevented by feeding the feces of rats kept on the same diet without sulfaguanidine. These findings indicate that the sulfaguanidine inhibits the bacterial synthesis of essential factors which are present in the natural B complex.

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POTASSIUM, SODIUM AND CHLORINE BALANCES OF PRE-SCHOOL CHILDREN RECEIVING MEDIUM AND HIGH PROTEIN DIETS[1]

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Sodium, potassium and chlorine are essential to the life of an organism. It is therefore surprising that there have been so few metabolism studies designed to determine the requirements of growing children for these elements. According to Stearns ('39) and also Shohl ('23, '39), sodium forms the largest portion of the total base of the body fluids and together with chlorine chiefly determines the osmotic equilibrium. Approximately 75% of the total body sodium functions in this manner and the remaining portion is found in a stable form in connection with bone and cartilage tissue. Chlorine occurs almost wholly in the extracellular fluids, the chief exception being the small amount contained in the red blood cells. Potassium functions mainly in relation to protein tissue, since approximately 72% of the total is contained in the muscle tissue. The remainder is found in other tissues, especially in the red blood cells and a small amount is present in the extracellular fluids. The assumption is generally made that all of the sodium and chlorine are contained in the extracellular and all of the potassium in the intracellular materials.

In previous publications, Hawks et al. ('37, '38, '40 and '42) reported studies showing the effect of increasing the protein

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content of the diet on the protein, calorie, calcium and phosphorus utilization of pre-school children. At the same time they also determined potassium, sodium and chlorine balances which will be discussed in this paper.

PROCEDURE

The details of the experimental procedure have been discussed fully in a previous publication (Hawks, Dye and Bray, '37), thus only brief details will be given here. Two groups of pre-school children received, first, a diet containing 3 and then one containing 4 gm. of protein per kilogram of body weight. In the first experiment, the 4-gm. protein diet contained additional milk, part of it skimmed to adjust the calorie value, meat and whole egg, while in the second experiment, egg white and gelatin were added and the amount of butter was reduced. All other foods comprising the two diets remained the same. In both experiments the conditions were very carefully controlled. The children consumed the food quantitatively and followed a definite routine each day. Experimental periods continued from 15 to 24 days. At the beginning of the experiment the three boys B, D, and V, were 55, 57 and 49 months old, and the two girls C and J were 37 and 38 months, respectively.

Sodium was determined by the Butler and Tuthill ('31) modification of the Barber and Kolthoff ('28, '29) uranyl zinc acetate method. Potassium was precipitated as sodium potassium sulfate according to the initial procedure of the Lindo-Gladding method (Peters and Van Slyke, '32). The potassium value was then determined by difference, using the sodium figures as previously determined. Chlorine was determined according to the Volhard-Harvey titration method (Peters and Van Slyke, '32).

RESULTS

Table 1 gives the balance data for all children on both experiments. The values are expressed as milligrams as well as milliequivalents per kilogram of body weight.

Experiment and child		Protein in diet	Potassium				Sodium				Chlorine			
			Range	Mean		Proportion of intake [1]	Range	Mean		Proportion of intake [1]	Range	Mean		Proportion of intake [1]
		gm.	mg.	mg.	meq.	%	mg.	mg.	meq.	%	mg.	mg.	meq.	%
I B	Intake	3	177–197	186	4.76		59.3–90.2	66.6	2.90		116–163	126	3.55	
		4	212–226	219	5.60		63.0–86.8	72.0	3.13		121–165	139	3.92	
	Feces	3	26–43	32	0.82	17.3	0.4– 1.9	1.1	0.05	1.6	2–3	3	0.08	2.2
		4	26–42	36	0.92	16.2	0.2– 0.9	0.6	0.03	0.8	3–4	3	0.08	2.4
	Urine	3	136–149	144	3.68	77.3	51.7–75.7	60.3	2.62	90.7	98–138	110	3.10	87.2
		4	163–183	171	4.37	78.1	52.1–74.7	65.1	2.83	90.3	95–127	113	3.19	81.7
	Apparent Retention	3	2–20	10	0.26	5.4	(−1.8)–12.9	5.2	0.23	7.7	3–23	13	0.37	10.6
		4	2–20	12	0.31	5.7	(−2.7)–16.3	6.3	0.27	8.9	13–34	23	0.65	15.9
I D	Intake	3	175–195	184	4.71		58.8–89.3	65.9	2.86		115–162	125	3.52	
		4	210–224	217	5.55		62.5–85.5	71.3	3.10		120–164	138	3.89	
	Feces	3	23–30	26	0.67	14.3	0.1– 1.8	0.7	0.03	1.0	2–4	3	0.08	2.2
		4	21–29	25	0.64	11.5	0.0– 0.7	0.2	0.01	0.3	2–3	3	0.08	1.9
	Urine	3	146–157	152	3.89	82.5	52.9–90.9	61.2	2.66	93.1	91–144	108	3.05	86.1
		4	175–185	180	4.60	82.7	57.8–76.7	65.3	2.84	91.6	99–132	112	3.16	81.3
	Apparent Retention	3	(−1)–11	6	0.15	3.2	(−2.8)– 7.6	4.0	0.17	5.9	8–21	14	0.39	11.7
		4	5–26	12	0.31	5.8	1.0–10.0	5.8	0.25	8.1	16–29	23	0.65	16.8
II V	Intake	3	199–206	203	5.19		58.7–60.4	59.4	2.58		107–114	110	3.10	
		4	198–208	202	5.17		58.8–62.7	60.3	2.62		107–111	110	3.10	
	Feces	3	27–34	31	0.79	15.2	0.2–00.4	0.3	0.01	0.5	1–3	2	0.05	1.8
		4	25–34	29	0.74	14.5	0.2–00.5	0.3	0.01	0.6	2–3	2	0.05	1.9
	Urine	3	156–161	160	4.09	78.7	51.0–53.3	52.0	2.26	87.6	100–109	102	2.88	92.9
		4	153–166	159	4.07	78.7	49.8–52.8	51.2	2.23	84.8	92–98	95	2.68	86.2
	Apparent Retention	3	10–17	12	0.31	6.1	5.4– 8.9	7.1	0.31	11.9	2–11	6	0.17	5.3
		4	9–21	14	0.36	6.8	6.8–11.4	8.8	0.38	14.6	9–16	13	0.37	11.9
II C	Intake	3	206–214	211	5.40		60.8–62.9	61.7	2.68		112–118	115	3.24	
		4	202–213	207	5.30		60.2–64.6	61.8	2.69		109–114	112	3.16	
	Feces	3	24–42	32	0.82	15.1	0.2– 0.5	0.3	0.01	0.6	1–2	2	0.05	1.5
		4	26–30	28	0.72	13.2	0.4– 0.5	0.4	0.02	0.8	2–3	2	0.05	2.1
	Urine	3	161–174	167	4.27	79.2	53.0–57.6	54.5	2.37	88.3	102–113	107	3.02	92.6
		4	162–173	166	4.25	80.4	47.9–57.8	53.9	2.34	87.2	93–102	98	2.77	87.2
	Apparent Retention	3	4–22	12	0.31	5.7	5.1– 8.8	6.9	0.30	11.1	3–13	6	0.17	5.9
		4	9–19	13	0.33	6.4	5.0–12.4	7.5	0.33	12.0	7–18	12	0.34	10.7
II J	Intake	3	193–202	197	5.04		56.9–58.7	57.8	2.51		105–111	108	3.05	
		4	190–198	194	4.96		56.0–60.4	57.8	2.51		103–106	105	2.96	
	Feces	3	32–38	35	0.90	17.6	0.7– 2.1	1.6	0.07	2.6	3–4	3	0.08	2.8
		4	35–39	36	0.92	18.8	1.0– 1.5	1.3	0.06	2.4	2–3	3	0.08	2.7
	Urine	3	147–154	151	3.86	76.6	46.4–52.6	50.9	2.21	88.0	90–104	99	2.80	91.6
		4	145–153	148	3.78	76.2	48.2–51.5	49.9	2.17	86.4	89–93	90	2.54	86.2
	Apparent Retention	3	8–17	11	0.28	5.8	3.4–11.6	5.3	0.23	9.4	1–18	6	0.17	5.6
		4	6–13	10	0.26	5.0	4.1– 7.6	6.6	0.28	11.2	11–13	12	0.34	11.1

[1] Percentage calculations were based on total daily figures in order to eliminate errors caused by rounding out the kilogram figures.

Potassium

Experiment I. Increasing the protein content of the diet from 3 to 4 gm. per kilogram had little, if any, effect on the apparent potassium retention for subject B, but caused an increase in the retention for child D. The slightly higher intake values on this diet may have been a factor in producing the increased retention. Fecal values were practically the same on the two diets, which made the amount of potassium absorbed higher in proportion to the intake on the 4-gm. protein diet. The increase in urinary potassium was also related to diet, for the percentage of the intake thus eliminated remained the same.

Experiment II. In this experiment the increase in the protein content of the diet apparently had no effect on potassium utilization. The mean intake values remained the same for individual children on the two diets and there was little variation in the figures from period to period. Fecal and urine values both remained practically the same for each child and there was no significant change in the apparent retention.

Sodium

Experiment I. Although there was a slight increase in sodium intake on the 4-gm. protein diet, the higher sodium retentions were probably due to the increase in the protein content of the diet because both milligrams of sodium and percentages of the intake retained increased. The children absorbed approximately 99% of the intake on both diets, but a smaller proportion of the intake was eliminated in the urine on the 4- than on the 3-gm. protein diet.

Experiment II. In this experiment the higher protein diet also caused an increase in the milligrams of sodium and in the proportion of the intake retained. There was little range in intake values and the mean figures were almost identical for each child. Feces contained practically no sodium so the differences in utilization on the two diets occurred after the sodium entered the body and were manifest by a lower amount and proportion of sodium excreted in the urine on the 4-gm.

protein diet. The higher fecal sodium for subject J no doubt was due to the fact that she had rather loose stools during the entire time.

Chlorine

Experiment I. The increase in the protein content of the diet caused a decided increase in chlorine retention in spite of the fact that the mean chlorine intake was slightly higher on the 4- than on the 3-gm. protein diet. Fecal outputs remained the same on the two diets, but the proportion of the intake eliminated in the urine diminished on the higher protein diet.

Experiment II. Chlorine intake was exceedingly constant on both diets, all figures ranging between 102 and 118 mg. per kilogram. The change in the protein content of the diet caused no difference in fecal outputs, but urine values decreased for each child and represented a smaller proportion of the intake on the higher protein diet. Thus without a doubt the higher protein diet caused an increase in the amount of chlorine retained.

DISCUSSION

Shohl ('39) states that in balance studies with sodium and chlorine it may take 2 weeks to establish equilibrium even on a constant diet and fluid intake. The data in the present experiment indicate that the children were in equilibrium. The fluid intakes and the diets, with the exception of the change in protein content, were constant throughout the entire experiment. The 10- or 12-day preliminary periods immediately preceding the medium protein diet were apparently adequate because there was little variation between the values during each 3-day period, and no more variation during the first periods than between those later in the experiment. In the second experiment, 9 days of the higher protein diet were considered as a preliminary period, but the average values for this period were almost identical with the average values during the last 15 days, and there was no more variation in

the results during the first 3-day period following the change in the protein content than there was during the later periods. It must be remembered, however, that there were practically no changes in sodium and chlorine intakes on the higher protein diets, and thus the change probably did not disturb sodium and chlorine equilibrium. Therefore it seems that any conclusion drawn from the results would be valid within the limits of any balance study.

Losses through the skin, which were not determined, probably reduced the quantity of these three minerals actually retained in the body. No figures are available for children of pre-school age, but Swanson and Iob ('33) found that infants from 3 to 6 months of age lost a considerable proportion of the apparent retention in this manner. The babies lost from 20 to 38% of the potassium, from 9 to 14% of the sodium, and from 13 to 18% of the chlorine. The children in the present study probably had relatively constant losses from day to day because the conditions of the experiment were so carefully controlled. They followed a regular routine for activity and rest, and the amount of clothing was varied to prevent excessive perspiration.

Since body tissue as a whole contains more potassium than sodium, the difference increasing with age, it seems desirable that the diet for growing children should contain a similar proportion. The Na/K ratio in milk is 1/1.5 by equivalents and should represent a desirable ratio for growth. In this study all of the diets had Na/K ratios similar to or higher than that for milk. In the first experiment, these ratios were 1/1.6 and 1/1.8 on the 3- and 4-gm. protein diets, respectively, and in the second experiment, they were 1/2.0 on all diets. Since the relationship was the same or nearly so, on the two levels of protein intake, the proportion could not have influenced the retention of these minerals.

In their studies on children, Macy and her associates (Souders, Hunscher, Hummel and Macy, '39; Hummel, Hunscher and Macy, '39; Hunscher, Hummel and Macy, '40) reported balance data for potassium, sodium and chlorine.

Intakes, as compared to those in the present study, showed a greater range in values and were lower for potassium, higher for sodium and similar for chlorine. In spite of these differences in intake, the children in both studies eliminated similar percentages of the intake of these three minerals in the urine and feces, and the retention figures were all within a similar range.

The actual retentions for the children in the present study ranged slightly above and below the figures for the daily accretions which Herter ('08) calculated from average body composition. He estimated that children should store an average of 0.36 meq. of potassium, 0.29 meq. of sodium and 0.25 meq. of chlorine daily. If his calculations were correct, the children were storing adequate amounts of these minerals to produce normal growth.

The children, as previously reported (Hawks, Bray and Dye, '38), gained weight during the entire experiment, but they gained from 2 to 6 times as fast on the 4- as on the 3-gm. protein diet. Being normal, they were undoubtedly producing both bone and soft tissue on both diets, but the composition of the growth was not identical. The higher nitrogen balances on the 4-gm. protein diet as well as the retention ratios between calcium, phosphorus and nitrogen (Hawks, Bray, Wilde and Dye, '42) indicated that the children produced a larger proportion of soft tissue than bone tissue on this diet than on the lower protein diet. The figures for potassium, sodium and chlorine substantiate the above conclusion as well as indicate that the soft tissue increase occurred both in muscle and extramuscle tissue.

Shohl ('39) and Peters and Van Slyke ('32), in reviewing the literature on this subject, say that potassuim is held within the tissue cell itself while sodium is found mainly in the interstitial fluids. Since the composition of the fluids remains exceptionally constant, it can be assumed that $(Na^+) + (K^+)$ when expressed in meq./kg. of water, represents the total amount of body fluid, and that the relationship between sodium and potassium indicates the proportion of extra- and

intracellular fluid. Thus on the basis of apparent sodium and potassium retentions, gains in body tissue can be expressed in terms of body fluids. Peters and Van Slyke, using the principle established by Gamble, Ross and Tisdall ('23), suggest the following formulae for calculating the liters of muscle and extramuscle fluid retained.

$$\frac{(Na^+) - 0.425\ (K^+)}{148} = \text{liters of extramuscle fluid.}$$

$$\frac{(K^+) - 0.017\ (Na^+)}{112} = \text{liters of muscle fluid.}$$

(Na^+) in muscle fluid $= 0.425\ (K^+)$. (K^+) in extramuscle fluid $= 0.017\ (Na^+)$.

(Na^+) of extramuscle fluid $= 148$ meq./l. of water.

(K^+) of muscle fluid $= 112$ meq./l. of water.

Calculations according to the above formulae (table 2) show that with the exception of child J, the cubic centimeters of fluid derived from both muscle and extramuscle sources increased on the high protein diet. Thus it can be said that the additional weight gains of the children on the higher protein diet were composed of both muscle and extramuscle tissue.

Both types of tissue, however, did not increase in the same proportion in the two experiments. In the first, muscle fluid

TABLE 2

Muscle and extramuscle fluid stored per kilogram of body weight.

EXPERIMENT	CHILD	PROTEIN IN DIET	MUSCLE FLUID		EXTRAMUSCLE FLUID		RATIO MUSCLE/EXTRAMUSCLE FLUID
			Amount	Increase with dietary protein	Amount	Increase with dietary protein	
		gm.	*cc.*	%	*cc.*	%	
I	B	3	2.29		0.81		2.83/1
		4	2.73	19.2	0.93	14.8	2.93/1
	D	3	1.31		0.72		1.82/1
		4	2.73	108.4	0.80	11.1	3.41/1
II	V	3	2.72		1.20		2.27/1
		4	3.16	16.2	1.53	27.5	2.07/1
	C	3	2.72		1.14		2.39/1
		4	2.89	6.3	1.28	12.3	2.26/1
	J	3	2.47		0.75		3.29/1
		4	2.28	—7.7	1.15	53.3	1.98/1

increased 19.2 and 108.4%, while extramuscle fluid increased only 14.8 and 11.1% for subjects B and D, respectively. In the second experiment, the reverse conditions were true. The percentage increases in muscle fluid on the higher protein diet were less than the percentage increases in extramuscle fluid. The ratios of the actual amounts of muscle to extramuscle fluid stored also show this same relationship. On the 4-gm. protein diet these ratios increased in the first, and decreased in the second, experiment. The fact that mineral intakes were slightly higher on the higher protein diet in the first experiment, and practically the same on the two diets in the second experiment, suggests that protein alone without the simultaneous addition of minerals cannot produce extra protein tissue as effectively as additional protein plus minerals. Thus potassium may be a limiting factor in the growth of muscle tissue. On the other hand, it is possible that the slight increase of 4.5 calories per kilogram on the 4-gm. protein diet in the first experiment may have had a protein sparing action and caused the increase in protein tissue.

Recent evidence indicates that it is questionable whether there is any chlorine in muscle cells, all of it being found in the extracellular fluids. Therefore the large increases in chlorine retentions on the higher protein diet lend further evidence to the conclusion that this diet caused an increase in extramuscle or extracellular fluid. Further, the greater proportional increase in chlorine retention for the children in the second, as compared to those in the first experiment, substantiated the conclusion that they produced a larger proportion of extracellular fluid on the 4- than on the 3-gm. protein diet.

Sodium and chlorine are so closely associated both within and without the body that it is often assumed that their utilization is parallel. They may, however, perform quite different and specific functions in the body. In this experiment, sodium and chlorine utilizations were not entirely parallel. The diet contained more chlorine than sodium and had no large excess of added salt. Fecal outputs for both minerals were all below 0.1 meq./kg., but chlorine values were slightly higher than

those for sodium. Urinary excretions of chlorine were also greater than those for sodium and followed intake figures to some extent, although the proportion of the intake excreted was lower on the 4- than on the 3-gm. protein diet. The relationships between sodium and chlorine retentions indicated that the children were not all producing the same type of extracellular tissue. The two boys in the first experiment and subject J stored more chlorine than sodium during the 4-gm. protein diet. All three children in the second experiment stored more sodium than chlorine on the 3-gm. protein diet, and subjects V and C stored equal amounts of the two minerals on the 4 gm. protein diet. The high chlorine retentions suggest that the children may have been producing some particular type of tissue, as blood cells, which contain large amounts of chlorine or that they may have been storing chlorine along with water in the skin or subcutaneous tissue.

SUMMARY

1. Five pre-school children received constant diets containing first 3 and then 4 gm. of protein per kilogram of body weight.

2. The amounts of potassium, sodium and chlorine which these children stored on both diets were similar to estimated daily accretions and to values which other investigators have obtained for children of similar age.

3. The 4-gm. protein diet produced little, if any, change in potassium retentions for some of the children, but caused a slight increase in others. This diet resulted in slightly higher sodium retentions and distinctly higher chlorine retentions for all of the children.

4. On the basis of potassium and sodium retentions, the percentage increase in muscle fluid on the higher protein diet in the first experiment was greater than the percentage increase in extramuscle fluid, while in the second experiment, there was a smaller percentage increase in muscle than in extramuscle fluid. At the same time the minerals in the diet increased slightly in the first, and remained the same in the

second, experiment. These facts suggest that an increase in protein without an increase in minerals did not cause as much muscle production as an increase in both protein and minerals and that potassium, since it is primarily found in muscle tissue, may be a limiting factor in muscle growth.

5. The increased chlorine retentions on the higher protein diets suggested that specific types of tissue may have been produced at that time.

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THE RIBOFLAVIN REQUIREMENT OF THE DOG[1]

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TWO FIGURES

(Received for publication June 24, 1942)

It has been demonstrated that the riboflavin requirement of the rat is increased markedly on a high fat diet (Mannering et al., '41). The present study was undertaken to determine whether the riboflavin requirement of the growing dog is influenced in a similar manner by the fat content of the diet. To this end, the riboflavin requirement of the growing dog, expressed in terms of micrograms of riboflavin per kilogram of body weight per day, was determined both on a high carbohydrate and high fat diet.

The recent recognition that choline is essential in the dietary of the dog (Schaefer et al., '41) has made it desirable to reinvestigate the symptoms of riboflavin deficiency as observed on highly purified diets containing ample amounts of choline. This is of special interest since the symptoms and pathology of riboflavin deficiency in the dog have been described by previous workers who have employed diets in which known amounts of choline were not supplied.

METHODS

Care of dogs. Mongrel pups which had been given a vermifuge were placed on the experimental diet at 6–7 weeks of age.

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[2] Commercial Solvents Corporation Fellow.

The pups were maintained on a milk diet from weaning until the time the experiment began. Fresh ration was supplied ad libitum. The daily food consumption of each dog was recorded and the animals were weighed daily. The dogs within the two groups employed were litter mates.

Rations employed. The high carbohydrate ration used is essentially that of Street and Cowgill ('39) and has the following percentage composition: sucrose, 66; casein (acid washed), 19; cottonseed oil, 8; cod liver oil, 3; and salts III (McKibbin et al., '39), 4.

As supplements the following crystalline vitamins [3] were fed: thiamine, pyridoxine and pantothenic acid at 100, 60 and 200 μg. per kilogram body weight per day, respectively; nicotinic acid and choline at 2 and 25 mg. per kilogram per day, respectively. The vitamins were prepared in a single solution of 20% ethanol at such a concentration that the solution could be fed at the rate of 1 cc. per kilogram of body weight on alternate days. As a source of the remaining filtrate factors required by the dog a butanol extract (Axelrod et al., '41) of liver powder [4] was fed in the ration at a level equivalent to 3% of the original liver powder. The riboflavin content of this preparation was less than 0.2 μg. per gram of liver powder as determined by a microbiological technique (Snell and Strong, '39). Riboflavin was fed on alternate days in a solution containing 250 μg. per cubic centimeter. The casein employed in this ration contained approximately 1 μg. of riboflavin per gram. Since the dogs consumed an average of 45 gm. of ration per kilogram of body weight per day, the daily intake of riboflavin supplied by the casein was 8.6 μg. per kilogram of body weight.

The high fat diet was prepared by isocalorically replacing a portion of the carbohydrate of the low fat ration with lard. The actual procedure was to replace 36 parts of sucrose with 16 parts of lard. To increase the consistency and palatability

[3] We are indebted to Merck and Co., Rahway, N. J., for generous supplies of the vitamins employed in this study.

[4] The liver concentrate powder (1–20) was supplied by the Wilson Laboratories.

of the ration the 8 parts of cottonseed oil were replaced by 8 parts of lard. The high fat ration had the following composition: sucrose, 30 parts; lard, 24; casein (acid washed), 19; salts III, 4; and cod liver oil, 3. The ration was prepared every few days and stored in the cold room. Under these conditions the ration was easy to handle and was very acceptable to the dogs. During the feeding of the high fat ration the thiamine supplement was fed at a level of 60 μg. per kilogram of body weight per day and the choline supplement at a level of 50 mg. per kilogram of body weight per day. The other experimental conditions remained as previously described.

Urinary riboflavin determinations. The dogs were placed in wire-bottom metabolism cages and the 24-hour urine samples were collected under toluene in dark bottles. Before storing in the refrigerator previous to assay the pH of the urine was adjusted to 6.8. The riboflavin content of the urine was determined by a microbiological method (Snell and Strong, '39).

Other determinations. The fat content of the liver was determined in the following manner. The freshly extirpated liver was oven-dried at 80°C. and the total lipids were subsequently extracted with chloroform (Channon et al., '37) in a continuous extractor for 24 hours. Blood was obtained by venepuncture and hemoglobin was determined colorimetrically by the method of Evelyn ('36).

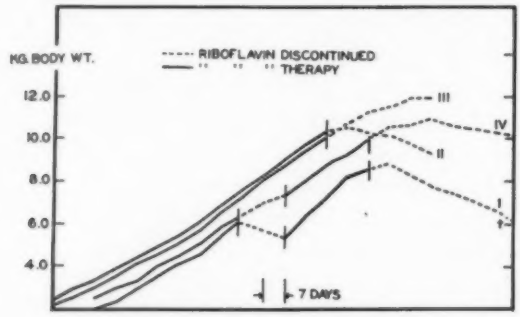
EXPERIMENTAL

A. High carbohydrate ration

Dogs I and II received 60, and dogs III and IV received 100 μg. of riboflavin per kilogram of body weight, respectively.

Dogs I and IV. After 47 days the riboflavin supplement was withheld. Dog I began immediately to decline in weight while dog IV continued to grow at the usual rate for 12 days and then reached a weight plateau (fig. 1). After 16 days without riboflavin, the dogs were given the supplement as before. In both cases a rapid growth response was observed.

Twenty-eight days later (91 days on experiment) the riboflavin supplements were again withdrawn. It will be noted from the figure that dog I continued to grow for 4 days and then began to decline rapidly in weight. On the other hand, dog IV continued to grow for some time and only after a 26-day period without riboflavin supplementation did the dog begin to lose weight. Dog I died 44 days after the last riboflavin supplement was given. The typical riboflavin deficiency symptoms were observed. Dog IV was still in good condition at this time.



+ indicates death of the animal.

Fig. 1 Growth of dogs on a high carbohydrate ration. Dogs I and II received 60, and dogs III and IV received 100 μg. of riboflavin per kilogram of body weight per day, respectively, during the periods indicated.

Dogs II and III. A good rate of growth was observed in both dogs for a period of 91 days (fig. 1). At this time the riboflavin supplement was discontinued. Dog II attained a stationary weight within 10 days and then began to lose weight rapidly. In contrast, dog III, receiving the 100 μg. supplement, continued to grow for 22 days. At this time the weight reached a constant level and no weight loss was observed even after 31 days without riboflavin.

The growth rates of all the animals in this group were similar and averaged 525 gm. per week during the period when riboflavin was being administered.

B. High fat ration

Four dogs were placed on the high fat diet and given supplements as follows: Dog V received no supplement, dog VI received 50 μg. of riboflavin per kilogram of body weight per day, while dogs VII and VIII received 100 μg. of riboflavin per kilogram of body weight per day.

Dog V. Although this animal received no daily riboflavin supplementation, a test dose of 1.35 mg. of riboflavin was injected subcutaneously after 18 days on experiment, and 1 mg.

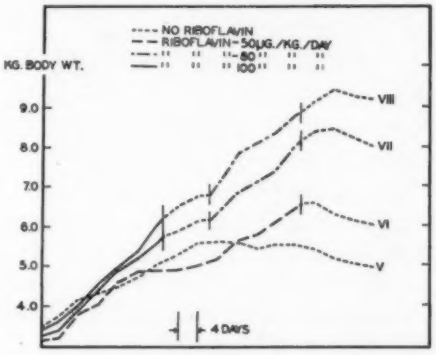


Fig. 2 Growth of dogs receiving a high fat diet supplemented with varying amounts of riboflavin.

of riboflavin was given orally by mistake on the twenty-fourth day. The dog grew fairly well for 32 days (fig. 2) and then reached a weight plateau. The weight remained constant over a considerable period of time and eventually the dog lost weight and died. This animal was observed to eat its feces and it is possible that this helped to maintain the dog for some time (Street et al., '41).

Dog VI. This dog received 50 μg. of riboflavin per kilogram of body weight per day for 53 days. Growth was maintained at a moderate rate until, after 53 days on experiment, the riboflavin supplement was withdrawn (fig. 2). At this time it will be noted that the weight of dog VI was about a kilogram

more than that of dog V but nearly 2 kg. less than the average weight of dogs VII and VIII receiving 100 μg. of riboflavin per kilogram of body weight per day. Growth ceased soon after the removal of the riboflavin supplement and a slow decline in weight ensued.

Dogs VII and VIII. These dogs received 100 μg. of riboflavin per kilogram of body weight per day for 25 days. At this time riboflavin was withheld and the animals were allowed to continue without supplement for 10 days until they appeared to reach a weight plateau (fig. 2). The dogs were then given a supplement of 80 μg. of riboflavin per kilogram of body weight per day and an immediate growth response was noted. After 18 days on this regimen the riboflavin supplement was discontinued. Growth soon ceased and both dogs maintained their weight for a short period of time before any appreciable weight losses were observed.

For comparison purposes the riboflavin intake for each dog in terms of food consumption has been calculated for the first 30 days on experiment and the values are given in table 1. The calculated figures expressing the riboflavin intake per gram of food consumed were obtained by dividing the total riboflavin intake during this period by the total food intake.

Urinary riboflavin excretion. The daily urinary riboflavin excretion was determined during the periods in which the dogs were receiving riboflavin supplements. The results appear in table 1.

Symptoms of riboflavin deficiency. After the completion of the riboflavin requirement studies, three of the dogs in each group were fed only their respective basal rations until the collapse syndrome appeared. Before the appearance of the collapse syndrome there elapsed intervals of time ranging from 6 to 13 weeks after the withdrawal of the riboflavin supplement. The fourth animal in each group was made an inanition control to one of the experimental mates. The control dog was given 100 μg. of riboflavin per kilogram of body weight per day and its food intake was restricted to that of its paired mate. The following observations are reported:

1. Fatty livers. All dogs on the basal diets showed typical friable, fatty, "yellow" livers upon autopsy. The fat content of the fatty livers ranged from 42.4 to 55.5% on the moisture-free basis. The livers of the two control dogs were normal in color and texture and contained 13.1 and 16.5% fat, respectively.

TABLE 1

Riboflavin intake and excretion.

DOG	DURATION OF EXPERIMENT	RIBOFLAVIN SUPPLEMENT		RIBOFLAVIN EXCRETION [1]
		Per kg. body weight per day	Per 100 gm. of ration consumed	Per kg. body weight per day
	days	*µg.*	*µg.*	*µg.*
I	45	60	140	4.8
II	40	60	158	4.8
	85			6.4
III	40	100	246	6.4
	85			47.0
IV	45	100	280	7.0
V	99	0	(—) [2]	1.5
VI	43	50	185	4.7
VII	43	100–80	244	5.6
VIII	50	100–80	254	6.5

[1] Each figure is an average of 3 or more values.

[2] Received test dose of 1.35 mg. subcutaneously after 18 days on experiment, and 1 mg. orally by mistake on 24th day. Was a coprophagist.

2. Anemia. The hemoglobin values varied from 9.9 to 12.3 gm. per cent. There was no correlation between the degree of riboflavin deficiency and the hemoglobin concentrations.

3. Muscular weakness. A muscular weakness of the hind quarters was observed in all of the deficient animals shortly before the appearance of the collapse syndrome. It did not appear to be due to inanition since neither of the control dogs was similarly affected.

4. Dermatitis. Five of the six basal dogs exhibited a dry, flaky dermatitis usually accompanied by a marked erythema on the hind legs, chest and abdomen.

5. Eye symptoms. In the dogs deprived of riboflavin, eye symptoms appeared in 4 to 9 weeks. A watery or purulent discharge from the eye accompanied by a conjunctivitis was first manifested. A few days later a vascularization of the cornea became noticeable and at the same time the nictitating membranes were pulled forward and the eyeball rotated dorsally in the manner described by Street et al. ('41). Three of the deficient dogs developed opacities of the cornea. The condition of dog I after 51 days on the basal ration was described by Dr. Mark E. Nesbit[5] as follows: "Bilateral conjunctivitis. Right eye, generalized grayness of the cornea with one area, round in shape, much more involved. Left cornea has some central grayness of the epithelial layer but otherwise clear. With the ophthalmoscope no blood vessels could be detected." No eye symptoms were observed in the control dogs.

6. Heart rate. In five of the deficient dogs heart rates of 140 to 190 beats per minute were observed a few hours before the collapse syndrome appeared.

DISCUSSION

From the results with the dogs on the high carbohydrate diet (fig. 1) it seems permissible to conclude that while there was no difference in the growth rates on the two levels of riboflavin employed (60 and 100 μg. per kilogram per day) there was a marked difference in the degree of riboflavin storage. When the high fat diet was employed, both 80 and 100 μg. of riboflavin per kilogram per day supported excellent growth. In one dog, the 50-μg. supplement did not prove to be sufficient for optimal growth. With either ration the riboflavin requirement for the growing dog can be tentatively placed between 60 and 100 μg. per kilogram of body weight per day. It should be pointed out that the ration is not completely riboflavin-free but that it actually contributes approximately 10 μg. of riboflavin per kilogram of body weight daily. Street and Cowgill

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('39) have suggested 25 μg. of riboflavin per kilogram of body weight per day as the riboflavin requirement for the adult dog. This value has been questioned since the food intake of the animals was restricted and the dogs lost weight during the experimental period (Axelrod et al., '40). Axelrod et al. ('41) have expressed the minimal riboflavin requirement of the growing dog as 200 μg. per 100 gm. of ration. In their work, 100 μg. of riboflavin per 100 gm. of ration proved to be inadequate while 400 μg. per cent was ample. These values are in fair agreement with the results of the present study. It is evident from table 1 that 50 or 60 μg. of riboflavin per kilogram of body weight per day corresponds to less than 200 μg. per cent while 80 to 100 μg. per kilogram per day amounts to more than 200 μg. per cent of riboflavin. Hughes ('40) has stated that the riboflavin requirement of the pig is between 1 and 3 mg. per 100 lbs. of body weight per day. Sebrell et al. ('41) have concluded, on the basis of riboflavin excretion studies, that the human riboflavin requirement is more than 35 and somewhat less than 60 μg. per kilogram per day. An estimate of the riboflavin requirement of the human adult can be obtained from our data with the following considerations. The function of riboflavin is related to its ability to serve as an integral component of a number of enzyme systems involved in the metabolism of carbohydrates, proteins and, possibly, fat. Thus, the need of the organism for riboflavin could be conditioned by the extent of the energy requirement. It is recognized that the energy requirement of a growing dog per kilogram of body weight is approximately twice that of an adult dog of the same weight. If the riboflavin requirement is in direct proportion to the energy requirement, it follows from our data that the riboflavin requirement is approximately 40 μg. per kilogram for an adult dog. However, a further correction is required since the food maintenance requirements of the adult dog vary with the weight of the dog. Our dogs weighed approximately 6 kg. and it can be noted from the maintenance prediction values of Brody, Proctor and Ashworth ('34) that the caloric requirement per kilogram

of a 50-kg. dog is 49/87 that of a 6-kg. dog. Again, presupposing a direct relationship between energy and riboflavin requirement, it follows that the riboflavin requirement of a 50-kg. dog would, therefore approximate $49/87 \times 40$ or 23 μg. per kilogram of body weight. On this basis the minimum riboflavin requirement of an adult human weighing 70 kg. would lie in the range of 1.6 mg. per day.

In general, it may be concluded that the riboflavin requirement of the growing dog fed the ration employed in the present study is not a function of the fat content of the ration. These results appear to be at variance with those previously reported for the rat (Mannering et al., '41). Recent work, however, may offer an explanation for this apparent difference between species. Mannering and Elvehjem ('42) have noted that the influence of fat on the dietary requirement of the rat for riboflavin is observed only when dextrin is employed as the source of carbohydrate; with sucrose as the carbohydrate component, no effect of fat was noted. It remains to be determined whether the substitution of dextrin for sucrose in the dog rations would make it possible to observe the effect of fat upon the riboflavin requirement.

It is of interest to note that the urinary riboflavin excretion of the dogs in the present study, during the period of riboflavin supplementation and rapid growth, is very similar to that previously observed (Axelrod et al., '41) in dogs maintained on a riboflavin deficient ration and receiving no riboflavin therapy. These values indicate a fairly complete utilization of the administered riboflavin during the period of rapid growth. The urinary excretion data lend further support to the conclusion that 60 μg. of riboflavin per kilogram of body weight per day is a minimal level. Thus, dog II, receiving this level of riboflavin therapy, showed only a small increase in the urinary riboflavin excretion per kilogram of body weight with the approach of maturity, while dog III, receiving 100 μg. of riboflavin per kilogram of body weight per day, showed a sevenfold increase in the urinary riboflavin excretion per kilogram of body weight (table 1).

The symptoms of riboflavin deficiency observed in the present study where known amounts of choline were added to the ration did not differ from those observed by previous workers. Thus, choline does not appear to have been a limiting factor in rations previously employed for the study of riboflavin deficiency in the dog. The "yellow," fatty livers previously associated with riboflavin deficiency by Sebrell and Onstott ('38) were also observed in the present experiments. No fatty livers were observed in the inanition control dogs, an observation which is not in agreement with that reported by Street et al. ('41). The development of ocular symptoms observed in the present experiments is similar to those previously reported in the riboflavin deficiency of other species.

Sebrell and Onstott ('38) have described a bradycardia associated with the collapse syndrome of riboflavin deficiency. In the present experiment the heart rates observed at the time of collapse were, with one exception, between 140 and 190 beats per minute. Street and Cowgill ('39) have stated that the heart rate was normal or rapid during the period of collapse syndrome.

SUMMARY

1. Under the experimental conditions described, the riboflavin requirement for the growing dog lies between 60 and 100 μg. per kilogram of body weight per day.

2. The isocaloric substitution of lard for sucrose does not increase the riboflavin requirement of the growing dog.

3. The following symptoms are associated with riboflavin deficiency: (1) fatty liver, (2) dermatitis, (3) muscular weakness of the hind quarters, (4) conjunctivitis, vascularization of the cornea, corneal opacities, and (5) tachycardia.

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METABOLISM AND GROWTH RATE OF RATS [1]

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TWO FIGURES

(Received for publication June 29, 1942)

This report presents data on day-to-day metabolism (oxygen consumption) of albino rats from birth to the age of 4 months, during which time they go through a series of striking changes, apparently not previously reported in the literature.

METHODS

Oxygen consumption was measured daily on each of three litters (seven, eight and nine per litter) from birth to weaning at 22 days.

Four males and four females, six of which were selected from the nine-rat litter and two from the eight-rat litter, were then measured individually from 24 to 121 days.

Beginning with the thirty-second day, fasting as well as non-fasting data were secured on the same eight rats, the fasts varying from 14 to 20 hours, depending on age. Between 32 and 45 days, only one male and one female were fasted at a time; thereafter all rats were fasted simultaneously.

Measurements were made in an 8-chamber Regnault-Reiset volumetric type apparatus similar in principle to the 4-chamber apparatus for fowls described by Winchester ('40). Windows in the chambers permitted the observation of activity, and as readings were taken every 10 minutes for more than 1 hour, observed activity could be eliminated from the

[1] Missouri Agricultural Experiment Station Journal Series No. 854.

results. Measurements were made at 30°C. between November, 1941 and April, 1942. The rats were housed at approximately 27°C.

RESULTS

The results are presented in figures 1 and 2 and table 1.

In figure 1, metabolism in terms of Calories per square meter of body surface, as given in table 1, is plotted against age

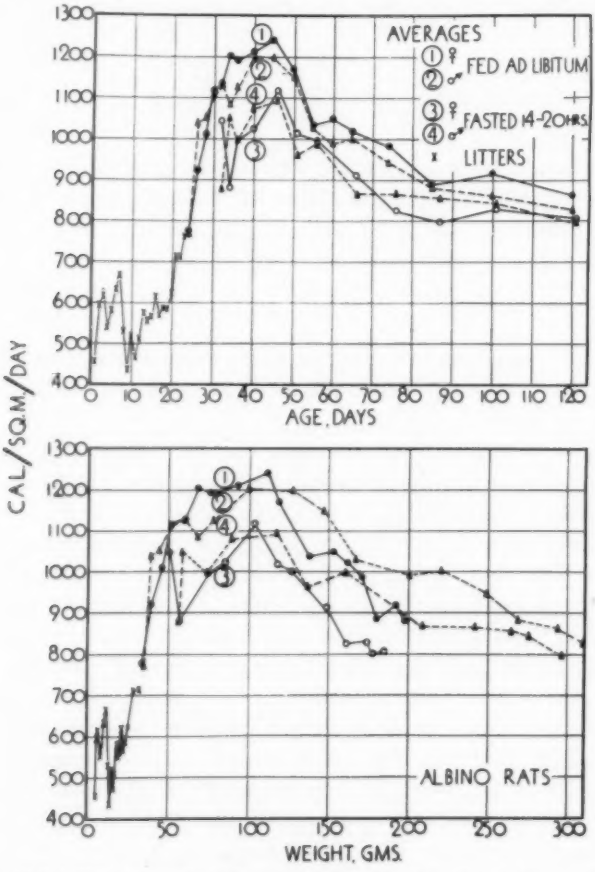

Fig. 1 Metabolism per unit surface area as a function of age (upper section) and of weight (lower section). Plotted from table 1.

TABLE 1

Growth and metabolism of albino rats.

(Basal values are shown on starred lines.)

LITTERS				FEMALES				MALES			
Age	Average per rat			Age	Average per rat			Age	Average per rat		
	Body weight	Cal.[1] Day	Cal./sq.m.[2] Day		Body weight	Cal.[1] Day	Cal./sq.m.[2] Day		Body weight	Cal.[1] Day	Cal./sq.m.[2] Day
days	*gm.*			*days*	*gm.*			*days*	*gm.*		
1	5.64	1.51	456	24	34.0	7.9	779	24	35.5	8.0	771
2	6.21	2.09	596	26	39.6	10.3	921	26	39.1	11.4	1038
3	7.07	2.31	614	28	46.0	12.4	1010	28	44.7	12.6	1053
4	8.37	2.33	552	30	52.2	14.8	1117	30	51.7	15.1	1114
5	9.74	2.70	581	32	60.8	16.5	1130	32	60.4	16.4	1130
6	10.7	3.11	635	*32	50.5	13.6	1045	*32	56.7	12.3	878
7	12.2	3.48	666	34	68.8	19.4	1203	34	68.4	17.6	1084
8	13.3	2.98	531	*34	57.2	12.4	880	*34	58.7	15.0	1048
9	14.2	2.55	437	36	76.6	19.8	1193	36	78.1	19.1	1126
10	15.5	3.22	520	*36	75.9	16.8	998	*36	74.2	16.5	995
11	16.3	2.97	466	40	93.3	23.2	1210	40	101.0	24.2	1204
12	17.4	3.41	512	*40	85.6	18.6	1022	*40	89.5	20.1	1080
13	18.9	4.06	578	45	112	26.7	1241	45	128	28.0	1198
14	19.8	4.05	559	*46	104	22.9	1119	*46	118	24.3	1094
15	20.8	4.24	564	50	119	26.2	1171	50	147	29.3	1149
16	21.7	4.74	616	*51	118	22.5	1015	*51	137	23.4	962
17	22.9	4.52	569	55	138	25.1	1035	55	167	28.4	1029
18	23.6	4.73	586	*56	127	23.2	998	*56	161	26.8	991
19	24.4	4.83	584	60	153	27.4	1048	60	201	30.8	991
20	25.4	5.29	624	65	162	27.6	1021	65	221	33.1	1002
21	28.9	6.53	713	*66	149	23.4	912	*66	209	27.6	867
22	32.1	7.02	714	74	171	27.7	987	74	249	33.6	946
				*76	162	22.4	824	*76	242	30.2	865
				85	180	25.6	884	85	269	32.9	882
				*87	178	23.0	800	*87	264	31.6	857
				100	192	27.7	917	100	293	33.9	862
				*101	174	23.6	829	*101	276	31.9	843
				120	197	27.1	880	120	310	33.7	826
				*121	185	23.8	806	*121	296	31.4	797

[1] The heat production was calculated on the assumption that 1 liter of oxygen had a heat equivalent of 4.7 Calories for the litters and 4.9 Calories for the older rats. A value of 4.7 Calories was also assumed for the fasted rats.

[2] Surface area was computed from the equation, surface area in square meters $= 0.0011$ (weight in gm.)$^{0.63}$ set up as a representative average relation from the surface area data of Carman and Mitchell ('26), Lee ('29) and Diack ('30).

in the upper section and against weight in the lower section. Prior to weaning, the curves appear irregular, varying around an average value of 576. Following weaning, maximum values of 1100 for fasting and 1200 for ad libitum feeding are reached at about the age of 45 days, or at a body weight of 100 gm. From 45 days to 4 months, the fasting values fall to 800, and the non-fasting values to about 850. The detailed characteristics of these curves have not been previously explored, apparently, although Mitchell and Carman ('26) reported higher basal values for rats between 32 and 40 days of age than for older rats up to 189 days, and other data in the literature bear on certain sections of the curves.

The rising and declining course of the age-curve of metabolism per square meter (upper section of fig. 1) is, in general, similar to that reported for other species; Riddle, Nussman and Benedict ('32) have reviewed the early literature. As our measurements were not continued beyond 4 months, they do not indicate whether there is a subsequent rise with increasing age as reported by Benedict and MacLeod ('29), or a continued fall with age as reported by Davis ('37). In dairy cattle (Brody, Kibler and Ragsdale, '41) the declining phase is less pronounced and is followed by a slight rise as the cattle reach a productive age.

It is perhaps significant that metabolism per square meter (fig. 1) rises during the period of rapid growth (during the age interval 24 to 45 days the body weights increase about 3.5 times), and declines as the growth rate declines (by the fourth month growth has practically stopped). Similar relations between metabolism per unit area and growth rate have been noted by Du Bois ('16) for children and by Riddle, Nussman and Benedict ('32) for young pigeons. Coleman and Du Bois ('15) also reported a basal metabolic level 16% above normal in adult typhoid fever patients in their second and third weeks of convalescence when they were rapidly regaining weight.

Sex differences in metabolism per unit area are small at all ages (upper section of fig. 1) but quite apparent for

given body weights above 100 gm. (lower section of fig. 1). The lower heat production of the females for given body weights is, perhaps, associated in part with their more rapid decline in growth rate. After growth has practically stopped, both sexes reach the same level.

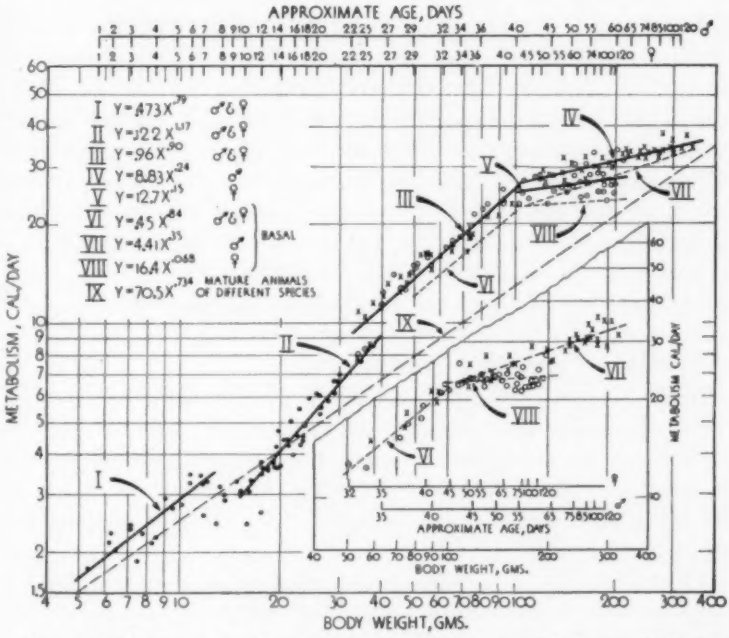

Fig. 2 Metabolism as a function of body weight in albino rats (individual data plotted on logarithmic paper). Basal data are shown in the insert. The other data represent ad libitum feeding. The curves for the various phases of growth represent the equation $Y = ax^b$ fitted to the data by the method of least squares.

When total metabolism is plotted against body weight on logarithmic paper (fig. 2) the data appear to be distributed in four fairly distinct segments. To each of these segments the equation $Y = ax^b$ was fitted with results given in figure 2. The meaning of the first break, between segments I and II, is not clear, but unpublished data obtained by a different

method on different animals and at a different time substantiate its reality. The break between segments II and III coincides with weaning, but may be due to a change in method of measurement, from entire litters in a chamber with no control over activity to individual rats in a chamber with elimination of activity.

The final break, although it may not be as definite as pictured, seems to be real and associated with a change in percentage growth rate. Preceding the break (at about 100 gm. body weight) metabolism increases with the 0.8 to 0.9 power of body weight (a 1.0% increase in weight is associated with an 0.8 to 0.9% increase in metabolism); following the break, during the period of declining growth rate, metabolism increases with less than the 0.4 power of body weight (the values for the various curves are given in fig. 2). On a percentage basis then, total metabolism increases more rapidly with weight during the period of rapid growth rate than during the period of declining growth rate.

Sex differences in metabolism at a given body weight become apparent after 45 days and, perhaps, likewise may be attributed, in part, to differences in rates of growth, the males being chronologically younger (see age scale at top of fig. 2) and growing more rapidly than the females of the same body weight.

Curve IX (fig. 2) represents the relation of basal metabolism to body weight in mature animals of different species within the given weight range (Brody, Proctor and Ashworth, '34). During the period of rapid-percentage growth from weaning to 45 days, the basal metabolism (curve VI) of the rats is considerably higher than that of mature animals of different species, but as the rats near their maximum body weights their metabolism is very little higher than that of other species.

SUMMARY

This paper presents day-to-day oxygen-consumption data on rats, from birth to 4 months, and brings out the following salient features:

The metabolic rate rises from about 550 Calories per square meter per day in early infancy (the low values perhaps being due to low muscle tonus and low endocrine activity) to 1100 basal or 1200 non-basal at the age of 45 days or at a body weight of 100 gm.; thereafter it declines to 800 basal and 850 non-basal, the decline in metabolic rate tending to parallel the decline in percentage growth rate. The decline in metabolic rate (as in growth rate) with increasing body weight is more rapid in the females than in the males.

When total metabolism is plotted against body weight on logarithmic paper, the resulting distribution exhibits "breaks" which apparently are associated, in part, with changes in percentage growth rate.

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EFFECTS OF PROLONGED DAILY TREATMENT OF NORMAL RATS WITH SALINE ANTERIOR PITUITARY EXTRACT

I. SEXUAL DIFFERENCES IN APPETITE, GROWTH AND ORGAN WEIGHTS [1]

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ONE FIGURE

(Received for publication June 13, 1942)

This investigation was undertaken for the purpose of elucidating the function of certain endocrine secretions regulating the distribution and disposition of nutrients and nutrient energy within the normal animal. Such information is especially necessary for an understanding of the utilization of dietary energy in normal nutrition. Toward this end observations have been made of daily injections of anterior pituitary extract (A.P.E.) as affecting appetite, growth, organ weights, protein metabolism and energy metabolism. Comparisons have been made of treated rats with untreated controls by paired and ad libitum feeding methods with both males and females as subjects.

This report is concerned specifically with the effects of the A.P.E. on appetite and growth, with emphasis on the different growth response of the sexes, and on the influence of appetite in this relation. The organ weights are considered,

[1] Authorized for publication on June 8, 1942, as paper no. 1107 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

Note: The experimental work on which this and the following related papers are based was designed and conducted by Max Kriss, deceased, with the collaboration of L. F. Marcy and Robert S. Bowman, the senior author having been delegated to present and to interpret the results.

incidentally, as affording possible clues to the causes of the differences in growth.

Differences in growth response of male and female rats subjected to continuing injections of A.P.E. were studied extensively by Evans and Simpson ('31). They concluded that the differences between the sexes resulted from a difference in tissue reaction and were independent of gonads and growth rates. Lee and Schaffer ('34) demonstrated that the growth response of female rats to A.P.E. was independent of its appetite stimulating effect. Their results were confirmed by Marx, Simpson, Reinhardt and Evans ('42) by the use of a growth hormone preparation free from pituitary "target organ" hormones. Thus, the divergence of response of males and females to A.P.E. has been recognized, and its specific growth effect has been demonstrated with females, but no corresponding investigation with male rats has come to the attention of the authors.

EXPERIMENTAL

The extract used in these investigations was prepared from the anterior lobes of beef pituitaries which were received frozen and kept in this condition until extracted. The extraction was carried out in a cold room as follows: 28 gm. of the glandular tissue was triturated with sand in a mortar surrounded by ice and stirred for ½ hour with 200 ml. of a 1% solution of sodium chloride. The mixture was centrifuged, and the supernatant extract transferred in 10 ml. portions to individual test tubes for daily use. The extract was kept frozen until used. Fresh batches of the extract were prepared as needed. The daily injections, which were made intraperitoneally, consisted of 1 ml. of the extract representing 140 mg. of the fresh anterior pituitary tissue. Each milliliter of the extract contained 1.5 mg. of nitrogen.

The effects of daily injections of this extract during a period of 12–14 weeks were investigated, first in 1939, with normal young male rats, and later, in 1941, the experimental program was repeated, in its essential details, with female rats.

Injections were started when the rats were 24 days old and weighed about 50 gm.

The rats were kept in individual cages and all received a stock diet of a nutritively complete commercial animal food.[2] Food consumption and body weights were recorded daily. Tap water was allowed ad libitum and, for the male rats, the water consumption was measured. The A.P.E. had no effect on water consumption in the males, and its effect was not determined with females.

The rats were grouped in litter-mate triplets, five triplets each of males and females being used for the main experiments. Of each triplet, one rat served as the control and two were treated with the A.P.E. One of the treated rats was pair-fed with the control and the other was fed ad libitum. In the experiments with the male rats the control rat received no injection, but in the experiments with females the control rat was injected daily with 1 ml. of 1% sodium chloride solution.

During the course of the experiments with males, rats other than the subjects of the main experiment were used to check the potency of the A.P.E. Six rats (three controls, three treated), fed ad libitum, were started during the ninth week. Since the A.P.E. gave no indication of deterioration under the conditions of preservation used, and results were duplicated with several shipments of the fresh frozen glands, similar tests of potency were not made in the experiment with females.

In the third, sixth and twelfth weeks of the experiment with the paired male rats, and in the fourth, seventh and thirteenth weeks with the female rats, growth continuity was interrupted temporarily with fasting periods in which food was withheld for 1 or 2 days for the purpose of metabolism measurements. The first fasting test with the treated male rats fed ad libitum was made during the second week. Otherwise, the fasting measurements for the three rats of each triplet were made simultaneously.

[2] Purina dog chow.

After 13 to 14 weeks the rats were killed with illuminating gas, and the weights of the livers, kidneys, hearts, adrenals and thyroids were obtained. With the female rats, the weight of the pancreas was also obtained.[3]

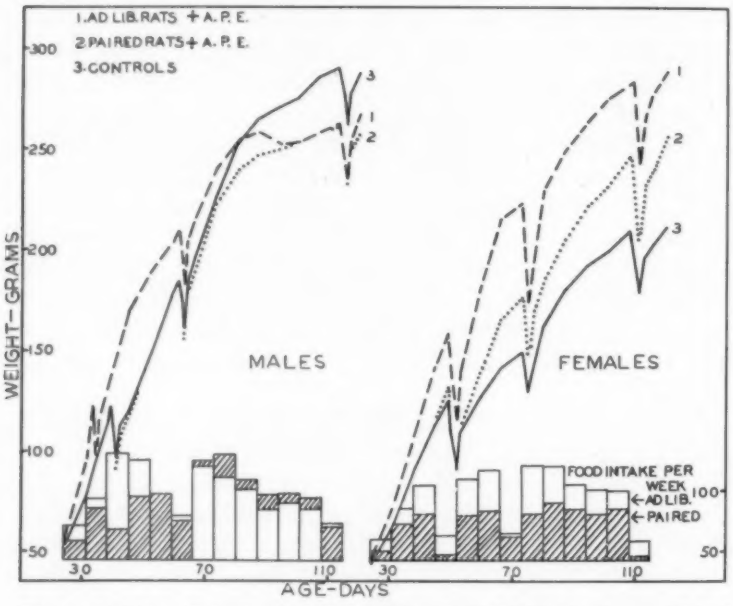

Fig. 1 Growth and food consumption of male and female rats as affected by daily injection of anterior pituitary extract. Breaks in growth curves represent fasting periods.

RESULTS

Appetite and growth

A notable difference in the response of the male and female rats to the A.P.E. was evident with respect to appetite and growth, especially after the treatment had been continued for several weeks. This difference between the sexes is conspicuous in the growth curves presented in figure 1, the breaks in the curves being caused by the fasting periods. The

[3] The authors are indebted to W. T. S. Thorp for dissecting out the organs.

essential features of the difference are illustrated by the data presented in table 1.

Both sexes responded to the A.P.E. initially with increased appetites, and the rats fed ad libitum outgrew the controls. The treated rats, which were pair-fed with the controls, were limited in food consumption by the latter, and their growth rates were essentially equal to those of the control rats for

TABLE 1

Effect of prolonged daily injection of A.P.E. on the relationship of food intake to weight gained by rats.

PERIOD	FOOD INTAKE		WEIGHT GAINED			FOOD FOR 1 GM. GAIN		
	Control and pair-mate	Ad lib.	Control	Pair-mate + A.P.E.	Ad lib. + A.P.E.	Control	Pair-mate + A.P.E.	Ad lib. + A.P.E.
				Males				
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Triplets								
First 4 weeks	309	423	95	93	135	3.3	3.3	3.1
Second 4 weeks	431	410	104	91	64	4.1	4.7	6.4
Third 4 weeks	398	364	34	21	6	11.7	19.0	60.1
Accessory rats [1]								
First 4 weeks	375	463	130		160	2.9		2.9
Second 4 weeks	518	496	102		74	5.1		6.7
				Females				
Triplets								
First 4 weeks	251	313	80	87	115	3.1	2.9	2.7
Second 4 weeks	306	414	38	53	71	8.1	5.8	5.8
Third 4 weeks	342	428	47	62	53	7.3	5.5	8.1

[1] Progress not interrupted by fasting periods.

the first 5 or 6 weeks. Following this period the responses of the males and females to the A.P.E. were distinctly different.

In the male rats no specific growth stimulation independent of food intake appeared at any time. The initial stimulating effect of the A.P.E. on appetite, which accounted for the extra gain in weight of the treated rats fed ad libitum, gradually disappeared, and during the final 7 weeks the appetites of the treated rats were depressed. It may be noted, in figure 1 and

table 1, that the food intake of the treated male rats fed ad libitum was actually less than that of the treated paired rats from the seventh to the twelfth weeks. Also, during this period three of the five treated paired rats consistently limited the food intake of their untreated control pair-mates.

Growth of the male rats receiving the A.P.E. was retarded during the same period in which appetite was depressed. These effects were partially independent, since the treated paired rats gained less weight than the controls after the seventh week. The treated male rats fed ad libitum lost their accumulated weight advantage in the ninth and tenth weeks, and completed the experiment on a weight par with the treated paired rats.

Male rats other than those of the main program, started under the same conditions 3 and 7 weeks later than the experiment proper, and not subjected to the fasting periods, also showed the reversal of response after the first 4 weeks. From the data in table 1 it may be noted that after the first 4 weeks all the treated male rats required more food per unit of gain in weight than did the controls. This difference was especially prominent in the last 4 weeks of the test, when the growth of the treated male rats fed ad libitum had practically ceased.

In contrast to the males, the female rats responded to the A.P.E. with increased appetites and growth, which effect continued to the end of the experiment. Partial independence of the appetite and growth-stimulating effects of the A.P.E. is evidenced after the fifth week by the extra weight gained by the treated rats which were pair-fed with the controls.

Some indication of a reactionary response by the female rats to the A.P.E. became evident in the last 4 weeks when the treated rats fed ad libitum gained less in weight than did the treated rats whose food intake had been restricted to that of the controls. In this period the food required per unit of gain in weight by the treated rats fed ad libitum was greater than that required by either the treated pair-fed rats or the controls. The appetite-stimulating effect of the A.P.E. in the treated rats fed ad libitum was still in evidence, but the

growth-promoting effect seemed to have waned during this period.

Organ weights

The organ weights reported in table 2 were obtained by adjustment of the original data to a standard body weight approximating the mean body weight of the rats when killed.

TABLE 2

The effect of prolonged daily injection of rats with A.P.E. on organ weights adjusted to standard body weight of 300 gm. by means of regression coefficients.

ORGAN	DURATION OF TREATMENT	REGRESSION ON BODY WEIGHT	ORGAN WEIGHT		SIGNIFICANCE OF DIFFERENCE [1]	REGRESSION ON BODY WEIGHT	ORGAN WEIGHT		SIGNIFICANCE OF DIFFERENCE
			Control	Treated			Control	Treated	
		Males				Females			
	days	*mg./gm.*	*gm.*	*gm.*		*mg./gm.*	*gm.*	*gm.*	
Liver	100	52.5	12.7	11.3	140	43.6	14.5	12.9	400
	72		14.3	11.5	90				
	40		14.3	12.4	40				
Kidneys	100	4.5	2.51	2.46	2	4.5	2.14	2.12	2
	72		2.69	2.38	40				
	40		2.68	2.48	8				
Heart	100	2.1	0.90	0.92	1	3.1	1.01	0.96	20
	72		0.98	0.95	1				
	40		0.98	1.02	1				
Adrenals	100	0.136	0.036	0.044	45	0.294	0.078	0.074	6
	72		0.038	0.050	18				
	40		0.039	0.053	17				
Thyroids	100	0.045	0.011	0.018	58	0.024	0.017	0.017	1
	72		0.017	0.020	350				
	40		0.018	0.016	1				
Pancreas	100					1.2	0.94	1.09	21

[1] Numbers indicate odds against difference occurring by chance alone.

The adjustments were made by means of regression coefficients computed from all measurements with either sex regardless of treatment. The individual adjusted values were compared as paired variates by the use of Love's ('24) modification of Student's table for estimating the odds against the differences occurring by chance alone. Customarily, odds of twenty or

more are considered to indicate a probable significant difference, while odds of one hundred or more indicate a highly significant difference.

The results representing the 40 and 72 days' treatment in table 2 apply to the male rats other than those in the main experiment. These observations offer some indication as to the time of appearance of the changes which seem to be significant. The experimental groups treated during 100 days include the five triplets each of males and females.

The liver weights of the rats treated with the A.P.E. were significantly lower than those of the controls in both males and females. This difference was evident in the rats treated during 40 and 72 days, as well as in those treated during 100 days.

A probably significant enlargement of the adrenals was evident in the treated male rats, but no difference in the weight of these glands was noticeable in the female rats.

The thyroids of the treated male rats were larger than those of the controls, but this difference resulted from a decrease in weight of the control rat thyroids rather than from an enlargement of these glands in the treated rats. Apparently the thyroid weights in the control rats decreased with maturation, although within the group (rats of same age) the weights of the thyroids showed a positive correlation with body weight. No difference was evident between the thyroid weights of the treated females and the controls.

Other differences of possible significance may be noted in the lower kidney weights of the treated male rats at 72 days, and the lower heart weights and enlarged pancreas of the treated female rats.

DISCUSSION

The results with the female rats corroborate, in a general way, those of Evans and Simpson ('31), Lee and Schaffer ('34) and Marx, Simpson, Reinhardt and Evans ('42). The results with the male rats differ from those of Evans and Simpson ('31) in that the rats of the latter did not show

the reversal of response encountered in the present investigation. Lee and Schaffer ('34) reported results with only two young male rats treated with A.P.E. during a period of 75 days. Neither of the two showed any significant growth stimulation above that recorded by their control pair-mates.

The depression of appetite and retardation of growth in the treated males were accompanied by a decrease in metabolic rate (results to be reported later). This would suggest either that thyroid activity was not related to the weights of the glands, or that the weight stasis of the thyroids in the treated rats resulted as a compensatory mechanism during the period in which the metabolic hormones of the A.P.E. were being counteracted. In this connection Turner and Cupps ('39) have suggested that the difference in growth rate of the sexes may be related to the difference in thyrotropic hormone production, since secretion of the thyrotropic factor increases during the period of most rapid growth in both sexes, but the female pituitaries contained only about half as much of the hormone as did those of the males.

Marx, Simpson and Evans ('42) reported that the thyrotropic hormone alone does not induce growth, but in association with the growth hormone it induces greater gains than does the growth hormone alone. Appetite effects were not recorded, so that the extent to which the extra gain resulted from increased intake or better utilization of food was not evident.

It is of interest that the liver weights in the treated rats of both sexes were significantly less than those in the controls. These results are in opposition to those of Lee and Freeman ('40), but are in apparent agreement with those of Marx, Simpson and Evans ('42) and Fraenkel-Conrat, Simpson and Evans ('42). According to the latter investigators a decreased liver weight is produced by a growth hormone, and an increased liver weight by thyroid stimulation. If this be true, the results reported herein would suggest that the effects of the growth hormone on liver weight and on growth were independent, since the growth responses of the males

and females were quite different, but the liver weight results were quite similar.

SUMMARY

The effects on appetite, growth and organ weight of normal male and female rats treated daily during a period of 12 to 14 weeks with a 1% saline extract of bovine anterior pituitary lobes have been investigated by paired and ad libitum feeding.

In male rats there was no specific growth stimulation independent of food intake. An initial appetite-stimulating effect accounted for the extra gains in weight of treated male rats fed ad libitum. This effect gradually disappeared, and during the final 7 weeks the appetites of the treated male rats were depressed, and growth was retarded independently.

In female rats appetite and growth were stimulated independently. Treated females pair-fed with controls showed extra gain in body weight after the fifth week of treatment, while those fed ad libitum grew at about the same rate as normal untreated male rats.

The liver weights of the treated rats of both sexes were less than those of the controls, on the basis of equal body weight.

The weights of the adrenals and thyroids in the treated male rats were larger than those of the controls, but no difference in the weights of these glands was evident in the female rats. The effect on adrenals in the treated males appeared to be a true enlargement, but the difference in thyroid weight resulted from a decrease in the weight of these glands in the control rats rather than from an enlargement in the treated rats.

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EFFECTS OF PROLONGED DAILY TREATMENT OF NORMAL RATS WITH SALINE ANTERIOR PITUITARY EXTRACT

II. PROTEIN AND ENERGY METABOLISM [1]

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In the preceding paper (Voris, Kriss, Marcy and Bowman, '42) sexual differences in the responses of normal male and female rats to prolonged daily treatment with anterior pituitary extract (A.P.E.) were reported with respect to appetite, growth and organ weights. The present paper reports the results of measurements of the utilization of food protein, the katabolism of protein during fast and the energy metabolism of these rats as affected by the A.P.E.

EXPERIMENTAL

The preparation of the saline A.P.E. and the general treatment of the rats have been described elsewhere (Voris et al., '42). Five litter-mate triplets of males and of females were used. Each triplet consisted of a control rat, its treated pair-mate, and a treated rat fed ad libitum. Injections were started when the rats were 24 days old and weighed about 50 gm.

Fasting metabolism measurements were made with the paired male rats in the third, sixth and twelfth weeks, and with the female rats in the fourth, seventh, and thirteenth weeks of treatment. The first fasting test with the treated male rats fed ad libitum was made during the second week. With this exception, the fasting measurements for the three rats of

[1] Authorized for publication on June 8, 1942, as paper no. 1109 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

a triplet were made simultaneously, and all triplets were fasted during the same week.

Collection of the fasting urine for nitrogen determination and the measurements of the respiration metabolism were started about 24 hours after the last feeding. Urine collection was continued for 24 hours with the male rats and for 48 hours with the female rats.

For 10 days, during the fifth and sixth weeks of the experiment, the food intake was standardized at 14 gm. daily for the males, and at 12 gm. daily for the female rats. After 3 days on the equalized feeding treatment, collection of urine and feces was made for 7 days, at the end of which time the respiratory metabolism was measured.

In a separate experiment two fasting tests about 10 weeks apart and one feeding test were made with six pairs of male rats which were given only three daily injections with the A.P.E.

Respiration measurements were made by the Haldane open-circuit gravimetric technic with an apparatus similar to that described by Forbes, Kriss and Miller ('34), with modifications reported by Kriss ('38). The total CO_2 and H_2O production and the insensible loss in weight of the rat during 7 hours' measurement were used for computation of the R.Q. The CO_2 production was determined hourly, but the CO_2 of the first hour, and any hour thereafter in which the activity of the rat was excessive, was excluded from the average. In the lighted chamber the rats were generally quiet, so the values determined for hourly heat production usually represented periods of 4 to 6 hours.

The O_2, CO_2 and heat involved in protein katabolism were computed from the urinary N, using the factors reported by Kriss and Voris ('37), i.e., 1 gm. of urinary N is equivalent to 5.96 liters O_2, 4.75 liters CO_2 and 26.71 Calories.

RESULTS

Disposition of food nitrogen. The results showing the effect of the A.P.E. on the disposition of food nitrogen are presented in table 1. The A.P.E. had no appreciable effect on the

TABLE 1

Urinary nitrogen excretion of fasting rats as affected by daily injections of anterior pituitary extract.

GROUP	MALES						FEMALES					
	No. of rats	Duration of treatment	Urinary N	Standard error of mean	Difference from control	Standard error of differences	No. of rats	Duration of treatment	Urinary N	Standard error of mean	Difference from control	Standard error of differences
		days	*mg./day*	±	*mg./day*	±		*days*	*mg./day*	±	*mg./day*	±
Controls	5		84	5			5		100	5		
Pair-mates + A.P.E.	4	15	128	10	44	11	5	25	159	16	59	16
Ad lib. + A.P.E.	2	10	90	12	6	13	4	25	141	9	41	10
Controls	5		112	10			5		104	5		
Pair-mates + A.P.E.	5	38	184	10	72	14	5	47	141	8	37	10
Ad lib. + A.P.E.	4	38	189	12	77	16	4	45	162	3	58	6
Controls	5		158	9			5		124	2		
Pair-mates + A.P.E.	5	88	175	16	17	18	5	86	167	9	43	10
Ad lib. + A.P.E.	4	88	222	15	64	18	4	86	155	9	31	9
Control	6		147	18								
Pair-mates + A.P.E.	6	3	118	16	—29	24						
Control	6		100	3								
Pair-mates + A.P.E.	6	3	88	6	—12							

apparent digestibility of food protein, as evidenced by the fecal nitrogen values which were essentially the same for the treated and the control rats of both sexes. Consequently the observed effects of the A.P.E. were referable entirely to the metabolism of the absorbed food nitrogen, as represented by the excretion of urinary nitrogen.

The female rats treated with the A.P.E. for 6 weeks retained over twice as much of the food nitrogen as did their control pair-mates with the same nitrogen intake. Male rats after 5 weeks of treatment retained 54%, and their control pair-mates 48% of the nitrogen absorbed. This difference was not statistically significant. However, male rats treated only three times with the A.P.E. showed a definite decrease in urinary N excretion. Thus, an initial food protein conservational effect of the A.P.E. appeared to have been somewhat counteracted by the sixth week of treatment in the male rats, but not in female rats. In our previous paper it was pointed out that the initial appetite-stimulating effect of the A.P.E. in male rats was being nullified at about this time.

Urinary N excretion of fasting rats. While the A.P.E. effected a conservation of food nitrogen by promoting its retention within the body, the rats treated with the extract for 2 weeks or more excreted considerably more N in the urine during fast than did the untreated controls (table 1). This effect was evident in all comparisons for both males and females in the three fasting periods. In only one comparison (paired male rats treated 88 days) is the difference of doubtful significance. On the other hand, with only three injections the average urinary N of the treated rats during fast was decreased. This was evident in both fasting tests made about 10 weeks apart on the same rats.

Energy metabolism of food. The effects of the A.P.E. on the energy metabolism of pair-fed rats nutritionally adjusted to constant food intake are shown in table 2.

The total heat production of male rats treated during 3 or 37 days, and female rats treated during 46 days, was increased by the A.P.E. However, in each case, the increase in total heat

TABLE 2

The disposition of food nitrogen, the respiratory quotients and heat production of pair-fed rats as affected by daily injections of anterior pituitary extract.

GROUP	DAYS TREATED	NITROGEN PER DAY				R.Q.		HOURLY HEAT		
		Intake	Feces	Urine	Balance [1]	Total	Non-protein [1]	Total	Protein [1]	Non-protein [1]
		mg.	*mg.*	*mg.*	*mg.*			*cal.*	*cal.*	*cal.*
Males										
Treated	3	432		128 ± 1		0.97	1.00	1025	147 ± 1	878 ± 42
Control		432		168 ± 2		0.99	1.05	878	183 ± 6	695 ± 21
Difference				—40 ± 2			—0.05 ± 0.02	147	—36 ± 7	183 ± 47
Treated	37	535	144	180	211 ± 16	0.99	1.03	1202	200 ± 17	1002 ± 46
Control		535	142	205	188 ± 8	0.99	1.04	1122	229 ± 9	893 ± 33
Difference					23 ± 18			80	—29 ± 19	109 ± 57
Females										
Treated	46	451	129	172	150 ± 6	0.96	1.00	1088	192 ± 7	896 ± 17
Control		451	133	247	71 ± 6	0.97	1.06	926	275 ± 6	651 ± 42
Difference					79 ± 8		—0.06 ± 0.016	162	—83 ± 9	245 ± 46

[1] ± figures are standard errors of means or differences.

was the resultant of a diminished protein oxidation and a relatively larger increase in the oxidation of carbohydrate.

None of the non-protein R.Q. values was less than unity. The R.Q.'s for the male rats treated during 37 days were not appreciably different from those of the controls, and fat synthesis was indicated in both groups. In the treated female rats and male rats treated during 3 days the non-protein R.Q.'s indicated carbohydrate oxidation only, whereas in their controls fat synthesis was evident.

Computation of the glucose equivalents of the extra non-protein heat produced by the treated rats in these groups, and the fat synthesized by the control rats, indicated that the treated rats were oxidizing about twice as much extra carbohydrate as the control rats were synthesizing into fat.

The effect of the A.P.E. was noticeably greater in the male rats treated for 3 days than in those treated for 37 days, and the effect in the females was greater than in either of the male groups.

Fasting energy metabolism. The fasting energy metabolism data for male and female rats are presented in table 3. The effect of the A.P.E. on the basal metabolism of the rats seemed to parallel the appetite effect with respect to the difference in reaction observed between the sexes.

In the treated male rats the total heat production of fast was increased initially, but this stimulatory effect was later counteracted, and after the twelfth week of treatment the heat production was lower than that of the controls.

In the treated female rats the total heat production was greater than that of the controls throughout the 12 weeks of treatment, though the difference in the twelfth week was not as extensive as that observed in the fourth and the seventh weeks.

Between the sixth and the twelfth weeks of treatment in both sexes the relationship of the fasting heat of the treated rats fed ad libitum to that of the treated pair-fed rats was reversed. The basal metabolism of the former, which was initially higher, became lower than that of the latter group by the twelfth week of treatment.

TABLE 3

Fasting heat production of rats at different times during treatment with A.P.E.

GROUP	NO. OF RATS	DAYS TREATED	WEIGHT	NON-PROTEIN R.Q.	TOTAL HEAT	PROTEIN HEAT IN PER CENT OF TOTAL	NO. OF RATS	DAYS TREATED	WEIGHT	NON-PROTEIN R.Q.	TOTAL HEAT	PROTEIN HEAT IN PER CENT OF TOTAL
	Males						Females					
			gm.		*cal./hr.*				*gm.*		*cal./hr.*	
Control	5	0	105	0.73	729	12.8	5	0	108	0.71	672	16.5
Pair-fed + A.P.E.	4	15	103	0.73	818	17.4	5	25	114	0.71	772	22.9
Ad lib. + A.P.E.	5	10	98	0.72	846	11.8	4	25	136	0.71	826	19.0
Control	5	0	176	0.73	898	13.9	5	0	147	0.71	776	14.9
Pair-fed + A.P.E.	5	38	171	0.73	886	23.1	5	47	173	0.71	943	16.6
Ad lib. + A.P.E.	5	38	200	0.72	949	22.1	4	45	203	0.71	997	18.1
Control	5	0	272	0.71	1039	16.9	5	0	194	0.72	807	17.1
Pair-fed + A.P.E.	5	88	246	0.72	941	20.7	5	86	229	0.71	947	19.6
Ad lib. + A.P.E.	5	88	258	0.72	905	27.3	4	86	264	0.71	836	20.7
Control	4	0	120	0.71	762	24.7						
Pair-fed + A.P.E.	4	3	121	0.71	903	14.8						

With only three injections of the A.P.E., the protein katabolism of the fasting rats was markedly decreased. After 10 days' treatment (male rats fed ad libitum) the fraction of the total heat derived from protein katabolism was practically the same for the treated and the control rats. In all other comparisons, when the treatment extended beyond 10 days the protein katabolism of fast was increased in the rats treated with the A.P.E.

The over-all statistics of table 4 give the cumulative results of all measurements in each group of rats for the three fasting periods and indicate the effect of the A.P.E. on the relationship of heat production to body weight. The data yielded by the rats injected for only 3 days with the A.P.E. were not included in these calculations, so the data reported represent the conditions prevailing during the time in which the treated rats were in a state of nitrogen plethora as evidenced by the excretion of excess urinary N during fast.

The correlation of hourly fasting heat production and body weight was considerably higher in the control rats than in those treated with the A.P.E. The regression coefficients expressing the average change in fasting heat production for unit change in body weight were 1.88 cal./hr./gm. in the male control rats, and 1.59 cal./hr./gm. in the female control rats. The digression from these values was most marked with the treated rat fed ad libitum, in which the regression of heat production on body weight was only 0.55 cal./hr./gm. for the males and 0.52 cal./hr./gm. for the females. In the treated rats pair-fed with the controls, the regression coefficient was decreased about 50% in the males, but only 10% in the females, the latter difference being non-significant.

Adjustment of the fasting heat production to a standard body weight of 200 gm., on the basis of the respective regression coefficients, indicated that the net effect of the A.P.E. on the total heat was negligible in the males, while in the females the total heat production was somewhat increased. However, the heat of protein katabolism was considerably increased in both sexes, and the values for this fraction of the

TABLE 4

Relation of fasting heat production to body weight of rats as affected by daily injections of A.P.E.

CATEGORY OF INTEREST	CONTROL RATS	PAIR-FED RATS + A.P.E.	AD LIBITUM + A.P.E.
Males			
Number of measurements	15	14	15
Mean body weight (range), gm.	184 (96–298)	179 (102–278)	185 (94–312)
Mean heat production (range), cal./hr.	889 (691–1187)	886 (801–1150)	900 (790–1100)
Correlation coefficient	0.93	0.56	0.45
Regression of total heat on body weight [1]	1.88 ± 0.20	0.97 ± 0.39	0.55 ± 0.30
Regression of protein heat on body weight	0.46	0.37	0.91
Regression of non-protein heat on body weight	1.42	0.60	—0.36
Total heat production at 200 gm. weight, cal./hr.	919	906	908
Protein heat production at 200 gm. weight., cal./hr.	138	191	221
Non-protein heat production at 200 gm. weight, cal./hr.	781	715	687
Females			
Number of measurements	15	15	12
Mean body weight (range), gm.	150 (104–209)	172 (108–247)	201 (125–299)
Mean heat production (range), cal./hr.	752 (644–860)	887 (736–1058)	886 (667–1045)
Correlation coefficient	0.82	0.72	0.27
Regression of total heat on body weight [1]	1.59 ± 0.31	1.44 ± 0.39	0.52 ± 0.16
Regression of protein heat on body weight	0.31	0.07	0.14
Regression of non-protein heat on body weight	1.28	1.37	0.38
Total heat production at 200 gm. weight, cal./hr.	832	927	885
Protein heat production at 200 gm. weight., cal./hr.	137	193	224
Non-protein heat production at 200 gm. weight, cal./hr.	695	734	661

[1] Cal. per hr. per gm. body weight.

total heat were remarkably similar for both male and female rats in each particular group. The extra protein katabolism of the treated rats fed ad libitum was larger than that of the treated rats pair-fed with the controls.

In the treated male rats the non-protein fraction of the fasting heat was decreased to about the same extent that the protein fraction was increased, so the net difference in total heat was negligible.

DISCUSSION

The saline A.P.E. used in the present investigation appeared to contain two principles affecting metabolism: one was active in promoting the increase in body substances (growth and conservation of protein); the other acted, directly or indirectly, as a stimulant of cellular metabolism.

After extended treatment an antagonistic agent developed, particularly in the male rats, which opposed both of these effects. The initial responses of the sexes were quite similar, but with progressive treatment the activity of the A.P.E. was counteracted in the male rats, and after 5 to 6 weeks the antagonistic agent became dominant over the A.P.E., as evidenced by the waning conservation of dietary protein, the decreased basal metabolic rate, the retardation of growth, and the depression of appetite.

The conservation of dietary protein persisted over an extended period of treatment with the A.P.E. The protein katabolism of fasting rats treated with the A.P.E. was decreased initially, but after 10 days the fasting protein katabolism was increased. These observations can be explained as the result of the accumulation of surplus protein. As the conservation of dietary protein persisted and the body stores increased, some of the extra supply was preferentially katabolized during fast, thus sparing the oxidation of fat as a source of energy for the increased metabolic rate. Consequently, after 2 weeks or more, the percentage of the total heat produced from protein by the rats treated with the

A.P.E. during fast was greater than that in the controls, and the protein sparing effect of the A.P.E. was obscured.

In view of the development of the antagonistic principles and the protein plethora, it appears that the limited treatment (3 days) with the A.P.E. afforded a more representative picture of the functioning of the anterior pituitary in the normal rat than did the extended treatment. The limited treatment under conditions of controlled feeding was sufficient to obviate the immediate adjustmental reactions and to reveal the essential regulatory nature of the principles in the saline A.P.E.

The increased heat production in the treated rats appeared to result from a true stimulation of cellular metabolism, since the extra heat was not correlated with body weight, and since the energy required for the accelerated metabolism was derived from the nutrient which happened to be most available at the time. Under normal dietary conditions carbohydrate provided the extra energy. During fast the extra energy was derived from fat, until the accumulation of surplus protein was such that protein katabolism increased and fat was spared.

The correlation and regression coefficients for the fasting heat production and body weight might be interpreted to mean that the weight changes (increase or decrease) induced by the A.P.E. involved the gain or loss of body substance which was less active, metabolically, than was the equivalent body substance in the control rats. This observation tends to confirm that of Lee and Gagnon ('30) and Kleiber and Cole ('39), with respect to the effect of the growth hormone on metabolism, but militates against the conclusion of the latter authors that the somatic adaptation of tissue metabolism is in correlation to body size.

As an alternate interpretation the low correlation and regression coefficients for the heat production and body weight in the rats treated with the A.P.E. may reflect a developing activity of antagonistic principles which counter-

acted the accelerating effect of the A.P.E. on metabolism in the earlier stages of treatment.

The results reported herein most closely agree with those obtained by Gaebler ('33) with dogs. In his subsequent investigations the calorigenic and protein sparing effects of the A.P.E. have been reproduced in thyroparathyroidectomized dogs (Gaebler, '35), and during phlorizin or pancreatic diabetes (Gaebler and Zimmerman, '39; Gaebler and Galbraith, '41). The results seem thus to be a direct effect of the A.P.E., rather than an indirect action mediated by the thyroid or pancreas.

In general, the results reported herein corroborate and extend the observations of Lee and Schaffer ('34), Schaffer and Lee ('35), Harrison and Long ('40) and Paschkis ('42) with respect to the effect of the A.P.E. on protein metabolism.

SUMMARY

The effects of daily treatment of normal young male and female rats with saline anterior pituitary extract (A.P.E.) over a period of 12 weeks was investigated with respect to the energy metabolism of protein and non-protein nutrients, and to the progressive changes of fasting metabolism relative to body weight and sex.

The total heat production under conditions of normal nutrition was increased by the A.P.E. The net increase was the resultant of a diminished protein oxidation and a relatively larger increase in non-protein oxidation.

In male rats the fasting heat production was initially increased, but this effect gradually disappeared and was finally reversed. In female rats the fasting metabolism was increased throughout the period of observation. The heat of protein katabolism during fast was initially decreased, but after 2 weeks or more of treatment it was greater than that in the controls for both sexes. This was attributed to the development of a protein plethora.

The regression of the fasting heat on body weight indicated that the changes in body substance induced by the

A.P.E. (or its antagonist, in the male rats) were not as active, metabolically, as were tissue changes in untreated rats.

The A.P.E. appeared to act as a specific stimulant of cellular metabolism and, at the same time, was effective in promoting an increase in body substance which was less energetic than that assimilated normally. In the male rats an antagonistic agent developed which opposed both of these effects.

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THE EFFECT OF VITAMIN B DEFICIENCY ON THE INTESTINAL ABSORPTION OF GALACTOSE IN THE RAT[1]

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The problem of intestinal absorption in vitamin B deficiency has attracted attention as being a possible explanation of the gastrointestinal dysfunction and anorexia that occur in such deficiency states (Pierce, Osgood and Polansky, '29; Gal, '30; Russell and Nasset, '41; Beams, Free and Glenn, '41; Harper, '42). The present study was undertaken in order to obtain further data on intestinal absorption in vitamin B complex deficiency.

EXPERIMENTAL

Albino and piebald rats reared in the department colony were placed on a diet having a percentage composition of alcohol extracted casein, 18; butterfat, 8; sucrose or alcohol extracted starch, 68; Mendel-Hubbell-Wakeman salt mixture ('37), 4; and cod liver oil, 2. Approximately one-half of the animals received the sucrose diet and one-half received the starch diet. Each experimental animal was "paired" with a litter mate of the same sex which received the same diet with the addition of 5 gm. of brewer's yeast per 100 gm. of diet.

[1] Presented before the Division of Biological Chemistry, American Chemical Society, at Memphis, April, 1942.

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The food intake of the control member of the pair was regulated so that its weight was kept nearly the same as that of the experimental member. Pairs of animals of different ages were purposely used. After from 30 to 45 days on the diet the experimental animals showed both marked loss of weight and deficiency symptoms. At this time the studies of intestinal absorption were carried out.

The animals were fasted for 24 hours prior to the absorption study, water being withheld during the last 12 hours. A volume of 25% galactose solution sufficient to supply 6.0 gm. of galactose per kilogram of body weight was placed in a small dish in the animal's cage. In all cases the animals voluntarily drank all of the sugar solution in less than 3 minutes. Exactly 1 hour after the animal had started to drink the solution the jugular vein was severed and a sample of 0.1 cc. of blood was obtained which was diluted with 2.4 cc. of distilled water and subsequently analyzed for galactose. The animal was then decapitated, the abdomen opened, hemostats clamped on the lower esophagus and the pylorus, and the gastrointestinal tract removed. These operations consumed approximately 1 to 1½ minutes. The contents of the stomach and intestine were then washed quantitatively into separate containers with hot water and diluted to volumes of either 250 cc. or 500 cc. A portion of each solution was then saturated with dry picric acid, filtered, and the picric acid filtrate analyzed for sugar by the method of Myers and Bailey ('16). The colors were read in an Evelyn photoelectric colorimeter. Control studies in fasting rats gave negligible quantities of reducing substances by this method. Galactose was estimated by fermenting the diluted blood with baker's yeast and subsequently determining the non-fermentable reducing substance. In the present study the figures obtained for blood galactose include those for the non-fermentable reducing substance of blood as well as for galactose. However, numerous studies have indicated that the non-fermentable reducing substance of rat's blood is fairly constant and always amounts to less than 50 mg. per 100 cc.

RESULTS

The results of absorption studies on thirty-eight pairs of animals in which the experimental member of each pair was deprived of the vitamin B complex are shown in table 1. This series contains both males and females and is made up of eighteen pairs of albino rats (indicated by W in the table) and twenty pairs of piebald rats (P). In both the deficient and control animals a considerable amount of the ingested sugar remained in the stomach. The average figure for the deficient rats is 35% of the ingested sugar, whereas the stomachs of the control animals contained 31% of the ingested galactose. The deficient rats had an average of 25% of the ingested sugar remaining in the intestine, whereas the control animals had an intestinal residue of 14% of the ingested galactose.

Absorption coefficients are expressed as milligrams of galactose absorbed per 100 gm. of body weight per hour. Some investigators maintain that the absorption coefficient can be more closely related to body surface than to body weight. In the present study this point is of no consequence since the deficient and control animals have approximately both the same body weight and body surface. The average absorption coefficient of the deficient animals is 244, whereas the average absorption coefficient of the control animals is 331. There is a considerable variation in absorption coefficient among the individual animals which is in part to be expected since the pairs are of different sexes, ages and weights and are of two different strains. In only six of the thirty-eight pairs does the absorption coefficient of the deficient animal exceed that of its litter-mate control.

Figures for blood galactose indicate an average value of 402 mg. per 100 cc. for the deficient animals, and 487 mg. per 100 cc. for the controls. In only three of the thirty-five pairs in which blood galactose estimations were obtained was the level of blood galactose of the deficient animal above that of its litter-mate control.

TABLE 1

The effect of vitamin B complex deficiency on absorption coefficients and blood galactose levels.

RAT			DEFICIENT ANIMALS						CONTROL ANIMALS						DIFFERENCE (CONTROL MINUS DEFICIENT)	
			Body weight		Galactose				Body weight		Galactose					
Pair	Sex	Strain	Initial	Final	In stomach [1]	In intestines [1]	Absorption coefficient	In blood	Initial	Final	In stomach [1]	In intestines [1]	Absorption coefficient	In blood	Absorption coefficient	Blood galactose
			gm.	*gm.*	%	%	*mg./ 100 gm./ hr.*	*mg./ 100 cc.*	*gm.*	*gm.*	%	%	*mg./ 100 gm./ hr.*	*mg./ 100 cc.*		
1	M	W	64	33	12	48	236	330	50	37	4	35	368	360	+132	+30
3	M	W	79	58	29	27	266	502	69	56	38	11	308	548	+42	+46
4	M	W	115	69	48	12	238	408	127	69	9	38	315	484	+77	+76
6	M	W	85	50	9	45	278	412	62	48	33	6	367	484	+89	+72
7	M	W	112	77	48	8	263	354	104	60	9	31	362	570	+99	+216
10	F	W	103	58	42	26	195	346	97	71	51	10	238	408	+43	+62
11	F	W	117	70	24	15	366	476	112	70	24	11	390	570	+24	+94
12	M	W	181	106	60	16	146	126	177	113	40	18	253	188	+107	+62
14	M	P	77	50	27	36	220	480	78	56	21	11	409	730	+189	+250
15	M	P	61	48	24	12	384	556	40	45	23	7	418	556	+34	0
16	M	P	60	45	21	17	374	552	41	46	40	10	304	488	—70	—64
17	F	P	51	43	16	10	447	708	45	42	26	9	394	580	—53	—128
18	F	P	70	44	31	48	129	328	72	55	46	8	275	440	+146	+112
19	F	P	74	52	49	21	184	368	59	55	14	13	435	700	+251	+332
20	F	P	45	29	26	36	224		48	37	18	15	400		+176	...
21	F	P	55	37	35	24	248	516	87	44	17	17	391	552	+143	+36
22	F	P	130	92	50	8	251	302	144	89	44	9	281	434	+30	+132
23	F	P	142	96	65	10	150	394	139	99	56	10	208	430	+58	+36
24	F	P	153	95	64	10	158	274	140	106	63	5	191	350	+33	+76
25	F	P	182	104	63	14	139	266	158	114	42	10	288	394	+149	+128
26	F	P	130	72	61	20	113	310	123	92	57	6	222	390	+109	+80
27	M	W	90	51	19	38	259	430	67	49	9	31	361	444	+102	+14
28	M	W	124	77	41	25	202		109	75	16	10	443		+241	...
29	M	W	73	49	12	35	317	452	78	43	35	12	316	390	—1	—62
30	F	W	83	47	18	36	275	400	93	53	27	10	380	560	+105	+160
31	F	W	67	43	14	40	277	448	60	49	17	10	437	502	+160	+54
32	F	W	111	70	40	30	182	346	112	83	41	9	303	488	+121	+142
33	F	W	73	46	10	56	204	452	65	47	20	12	406	570	+202	+118
34	F	W	88	59	16	43	248	416	88	57	24	14	368	502	+120	+86
35	F	W	72	52	52	9	237	390	72	43	11	16	440	458	+203	+68
36	F	W	59	39	23	24	321	372	74	42	6	31	374	580	+53	+208
38	M	P	43	35	20	30	300	502	57	45	6	9	510	766	+210	+264
41	M	P	192	119	42	22	221	318	203	118	53	17	175	324	—46	+6
42	M	P	124	87	59	10	188	302	100	90	54	6	241	400	+53	+98
43	F	P	37	30	9	38	317	484	32	30	29	15	334	498	+17	+14
45	F	P	107	74	41	13	274		92	74	50	13	221		—53	...
46	F	P	93	61	48	17	210	372	93	71	47	11	252	494	+42	+122
47	F	P	87	58	48	13	230	364	98	67	57	8	214	390	—16	+26
Average			95	61	35	25	244	402	91	64	31	14	331	487	+87	+85

[1] Fraction of ingested sugar found in the organ.

DISCUSSION

The technic employed in the present study differs somewhat from that popularized by Cori ('25) although none of the variations are necessarily original. In some rats forced feeding by means of a stomach tube causes marked excitement accompanied by violent struggling. It is well-known that in humans such states of excitement are accompanied by alteration in gastrointestinal motility, and this is also undoubtedly true in rats. By having the rats spontaneously ingest the sugar any excitement is eliminated. Separate analyses of unabsorbed sugar in the stomach and intestine were made since it was felt that information regarding gastric evacuation of the sugar solution could be gained by this means and it was appreciated that this factor might greatly contribute to any results obtained. The amount and concentration of sugar fed have been reduced as much as possible. It is well-established that solutions of high osmotic pressure are not readily evacuated from the stomach, and in experiments which we have conducted on human subjects ingestion of strong sugar solutions frequently resulted in nausea and vague gastrointestinal distress. We feel that reduction in amount of sugar fed to such a level that the animals will voluntarily ingest the entire amount is desirable and outweighs advantages that would be afforded by a longer experimental period which would necessitate forced feeding of the rats.

Galactose was selected as a test sugar in these experiments since this sugar has been employed in this laboratory in indirect studies of intestinal absorption in dogs and humans (Beams, Free and Glenn, '41; Free, Leonards and Myers, '42). This sugar is one which is selectively absorbed from the intestine at a rate greater than can be accounted for by simple diffusion. The results of Cori ('25) indicate that glucose and galactose have quite similar rates of absorption.

Cowgill, Deuel, Plummer and Messer ('26) noted that gastric atony was present in dogs with advanced vitamin B deficiency. Marked decrease in the rate at which vitamin B

deficient rats evacuated barium sulfate from the stomach was observed by Gal ('30) and by Gershon-Cohen, Shay and Fels ('41). Heublein, Thompson and Scully ('41) have reported that vitamin B complex deficiency caused marked retardation in gastric motility and moderate delay of small intestinal motility, and in our laboratory we have observed very marked gastric retention in dogs suffering from acute symptoms as a result of vitamin B complex deficiency. However, in the present study the data on gastric and intestinal residues of unabsorbed sugar indicate that, on the average, gastric emptying in the deficient rats has occurred at approximately the same rate as in the pair-fed controls since the average gastric retention in the deficient animals is 35% of the ingested dose, and in the controls, 31%. The residue of unabsorbed sugar in the intestines of the two groups indicates that the deficient animals did not absorb the galactose as rapidly as the control animals even though it was present in the intestine and available for absorption.

The actual values of the absorption coefficients are considerably higher than those obtained by Cori ('25) for galactose in control animals. It would appear that two factors contribute to these higher levels. In the first place, the average weight of the animals at the time of the absorption studies is considerably less than the weight of the rats employed by Cori. Furthermore, the rats in the present study had decreased in weight on an average of 30 gm. during the depletion period so that the ratio of intestinal surface to body weight was increased. The second factor which probably contributed to the greater absorption rate in our animals was the fact that the rats voluntarily ingested the sugar and did not suffer any fright or excitement as a result of the gavage.

Comparison of blood galactose elevation with the absorption coefficients indicates that the level of blood galactose 1 hour after ingestion of the galactose solution is a measure of the rate of absorption during the period. Different animals will have somewhat different rates of metabolism and excretion of the sugar but in general high blood galactose figures

are associated with high absorption coefficients and low blood galactose figures are associated with low absorption coefficients. In the thirty-five pairs of animals where blood galactose and absorption coefficients were obtained, there were five pairs in which the absorption coefficient of the deficient animal exceeded that of its control litter mate. In three of these pairs the level of blood galactose of the deficient animal also exceeded that of its litter mate. In only two pairs (nos. 41 and 47) did the values of blood galactose not correspond with the absorption coefficients.

In the present study no attempt was made to determine which member of the vitamin B complex contributed to the impairment in intestinal absorption. The animals in the present study were somewhat analogous to humans who subsist on vitamin B deficient diets which, rather than being deficient in a single member of the vitamin B complex, are deficient in most of them. However, they differ from human vitamin deficiency states in that restriction of vitamin B complex was complete in the animals and they developed acute symptoms in a short time. At the present time further studies are being made to ascertain which member or members of the vitamin B complex may be specifically involved in intestinal absorption.

Results of the present study do not indicate whether vitamin B complex deficiency has a specific action on the physicochemical processes of absorption, or whether the effect is secondary to changes in the circulation or motility of the intestine.

SUMMARY

Studies of the intestinal absorption of galactose were carried out in thirty-eight litter-mate pairs of rats in which one member of the pair received a diet deficient in the vitamin B complex, whereas the other member received the same diet supplemented with brewer's yeast. Intestinal absorption was determined by analysis of the unabsorbed sugar in the gastrointestinal tract 1 hour after its oral ingestion. The rate of intestinal absorption of the pair-fed litter-mate controls aver-

aged 35% more than that of the deficient animals. Analysis of the gastric residue in the animals indicated that the results were not affected by the rate of gastric emptying since this was approximately the same in the two groups of animals. Blood galactose levels 1 hour after the ingestion of the sugar were used as indirect measures of intestinal absorption. The blood galactose levels also indicated an impairment of intestinal absorption in the vitamin B complex deficient animals.

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MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1943 Award of $1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at Cleveland on April 7, 1943.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1942 must be in the hands of the Secretary by January 10th, 1943. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

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